REMARKS

Reconsideration of the captioned patent application in light of the above amendments and the following discussion is courteously requested.

Claims 1-43 were presented for examination. Applicants gratefully acknowledge the Examiner's thorough examination of the application, and the many helpful suggestions set forth in the Office Action. Several of the claims have been amended above. A set of claims is attached hereto marked to show the changes.

The Examiner set forth a requirement for restriction under 35 U.S.C. 121 and 372. The Examiner asserts Groups I, II and III, as delineated in the Office Action, are not so linked as to form a single general inventive concept as required by PCT Rule 13.1. Applicants, through their attorney during a telephone interview with the Examiner on 2/5/01, provisionally elected the subject matter of Group I, Claims 1 (in part), 2-16 and 25-43 (in part), for prosecution on the merits. This election is hereby affirmed. The provisional election was made with traverse, but after reviewing the Examiner's cogent arguments supporting the requirement for restriction, Applicants hereby withdraw the traversal. In an effort to progress prosecution of the elected subject matter to speedy allowance, the non-elected subject matter of Claims 17-24 (Group II) has been cancelled. This cancellation is done with reservation of the right to file one or more divisional applications embracing the cancelled subject matter, and Applicants in no way relinquish their claim to the cancelled subject matter. The remaining claims have been amended so as to read only on the Group I subject matter (namely, compounds of Formula I where G and Z both are nitrogen). All rights are reserved in all cancelled subject matter, pending filing of divisional applications.

Inventorship of the elected Group I subject matter has been reviewed, and Applicants hereby confirm that all the originally named inventors made inventive contributions to the now elected subject matter of Group I.

The Examiner objected to the Specification for several reasons. First, no Abstract was present. Applicants submit herewith, on a separate sheet, an Abstract. The Examiner noted that certain formulas in the Specification (on pages 32, 37, 40 and 41) are not consistent with general Formula I. The Specification has been amended by adding structures that are within general Formula I. The added structures are fully supported in the Specification, especially on page 14, and no new matter is added. Applicants courteously submit that the amended Specification and enclosed Abstract obviate the basis for objection to the Specification.

Claims 1, 10, 12, 15, 25, 26, 30 and 34 stand rejected under 35 U.S.C. 112, second paragraph. Specifically, Claims 1, 25, 26, 30 and 34 fail to recite the proper electronic charge on the nitrogen atom in the definitions of R^1, R^2, R^{10}, T and Q. Claims 25, 26, 30 and 34 are cancelled, thereby rendering their rejection moot. Claim 1, and claims dependent thereon, have been amended to show the proper electronic charge.

Further, the Examiner noted that R^9 in various of the claims is unclear for calling for "carbonyl", "thiocarbonyl" or "imine". These terms have been deleted and replaced by the specific chemical structures to which they refer.

Claims 10 and 12 are said to lack antecedent basis since X in Formula I is NR^{10}, not $NHC(=O)-R^4$. The claims have been amended to correct the structures, thereby finding proper antecedent basis. Claim 15 is said to lack antecedent basis in Claim 1 because R^9 is not defined therein to include "oxo". Claim 1 has been amended to include the requisite antecedent basis. Support is found in Claim 15 as filed, as well as in the Specification on pages 88-90 and in Table 3b on page 97. No new matter is added by the amendment to Claim 1.

Applicants believe the amendments to the claims overcome the rejections under 35 U.S.C. 112, and request withdrawal thereof.

Claims 25-36, 38, 40 and 41 stand rejected under 35 U.S.C. 101 as drawn to methods of use that allegedly are not supported by a well-established utility. The claims are also rejected under 35 U.S.C. 112, first paragraph, as allegedly unsupported by a full description of "how to use". Claim 25 is cancelled, thus rendering its rejection moot. Claim 26 is drawn to a method for inhibiting a cyclin-dependent kinase enzyme. Claims 27-29 are directed to inhibiting specific cyclin-dependent kinase enzymes. Claims 30-36 are drawn to methods for inhibiting various tyrosine and/or serine kinase enzymes. Claim 37 is amended to read on a method for treating vascular smooth muscle cell proliferation using a compound of Claim 1 (as amended). Claims 38 and 40 are cancelled, thus rendering their rejection moot. Claim 39 is drawn to a method for treating angiogenesis using a compound of Claim 1 (as amended). Claim 41 is drawn to a method for inhibiting the kinase enzyme known as "wee-1".

Applicants respectfully submit that the Specification discloses not only believable utilities for the claimed compounds, but also a full and clear teaching of how to use them. As noted on pages 1 and 2 of the Specification, kinases are enzymes that are naturally occurring in mammals, and are well established to be responsible for a number of diseases that result from uncontrolled cell proliferation. Several known compounds that inhibit cyclin-dependent kinases have been shown to inhibit cancer cell growth in vivo, and as noted on page 1, such compounds are routinely used by the National Cancer Institute as a reliable assay for potential clinical cancer agents. In addition, the kinase enzymes have been well established to be involved in promoting growth of various cell forms, including fibroblast and vascular cells. Compounds that inhibit the activities of these naturally occurring enzymes will be useful to clinically treat conditions resulting from uncontrolled cell growth, conditions such as angiogenesis, psoriasis, and atherosclerosis.

A great deal of research into cyclin-dependent kinases is currently ongoing at pharmaceutical companies around the world. Much of this work is summarized by Fry and Garrett in "Inhibitors of cyclin-dependent kinases as therapeutic targets for the treatment of cancer", Current Opinion in Oncologic, Endocrine & Metabolic

Investigational Drugs 2000, 2(1):40-59 (copy enclosed). The authors note that "...the rationale to inhibit certain CDKs as a specific approach to cancer chemotherapy has become progressively stronger as research in this area continues to identify tumor-selected abnormalities in the cell cycle that appear to contribute to the development and progression of cancer (page 40, first paragraph).

Sielecki et al., in J. Med. Chem., 2000, Vol. 43, No. 1, pp 1-18 (copy enclosed), discloses that inhibitors of cyclin-dependent kinases are useful in the "treatment of proliferative diseases, including cancer" (p. 2), and that cell cycle alternations have also been contributing factors in "certain neurodegenerative diseases,...and several viruses" (page 5, column 1). Sielecki et al. describe several chemical inhibitors of cyclin-dependent kinases, some of which are being developed clinically for treating myeloma, gastric carcinoma, and cell lymphoma (see page 7, col. 2). Indeed, compound 25, discussed on page 10, is somewhat related to some of the now claimed compounds, and it is being actively studied as an anti-cancer drug. This is clear evidence that compounds of the general type now claimed are useful.

Still another article expressing the usefulness and importance of cyclin-dependent kinase inhibitors is by Sherr in Cancer Research, Vol. 60, No. 14, 2000, pp 3683-3987 (copy enclosed). Indeed, Sherr states, regarding cell cycle transitions and cyclin-dependent kinases, that "...good science will lead to good medicine." (p. 3693).

Applicants have established, in the Specification on pages 90-103, that the instantly claimed compounds do in fact inhibit the biological action of several kinase enzymes. Tables 1-2 provide conclusive evidence that a wide variety of invention compounds inhibit a broad representative group of cyclin-dependent kinase enzymes. Tables 3 and 4 provide clear data establishing the invention compounds inhibit the tyrosine kinase enzymes known as fibroblast growth factor and platelet derived growth factor. These are real enzymes, occurring in living biological systems. Inhibition of these real enzymes is a biological activity that is real, believable, and potentially useful in clinical treatment of life-threatening diseases. While the data presented in Tables 1-6

(pages 92-100) was generated in standard and well accepted in vitro assays, the data in Table 7 is even more convincing, given that it shows the ability of invention compounds to actually inhibit the uncontrolled proliferative growth of actual biological cells, namely human umbilical vein endothelial cells. This data clearly establishes that the invention compounds are inhibitors of living cell growth. These data translate directly to useful clinical implications. Applicants respectfully submit that the data presented in the application are more than adequate to establish a believable utility of the claimed compounds within the meaning of 35 U.S.C. 101.

Moreover, the Specification complies fully with the requirement of 35 U.S.C. 112 to teach "how to use" the now claimed compounds. Page 2 sets forth that the compounds are useful for several clinical indications, including atherosclerosis, restenosis, psoriasis and angiogenesis. Page 90 states clearly that the compounds are potent inhibitors of kinase enzymes, and as such are useful to treat diseases resulting from uncontrolled cell proliferation. Pages 100-101 establish with working data that the compounds do in fact inhibit the uncontrolled growth of human cells. Page 103 sets forth that the subject compounds can be administered to mammals by the oral or parenteral routes, and Examples 75-80 on pages 103-105 give specific examples of typical pharmaceutical formulations. Pages 15-18 describe the various pharmaceutical forms that the instant inventions can take, and the typical excipients utilized to produce those forms. Page 18, at lines 17-26 describe in detail exactly what doses of invention compounds will be administered. Accordingly, Applicants submit that the Specification provides a full and clear description of how to use the claimed invention, and that the rejection under 35 U.S.C. 112, first paragraph, is in error and should be withdrawn. Such action is requested.

Claims 1, 2, 7, 8 and 43 stand rejected under 35 U.S.C. 102(g) as allegedly anticipated by Harris et al., U.S. Patent No. 6,150,373. The '373 patent is a reference as of its filing date, namely October 21, 1999 (the United Kingdom priority documents are not available to set the effective reference date). Applicants' application has a priority date of May 26, 1998, more than one year prior to the date of the '373 reference.

Accordingly, the '373 patent is not available as a reference against the instant application. Withdrawal of the rejection under 35 U.S.C. 102(g) is requested.

Applicants gratefully acknowledge the Examiner's indication that Claims 9, 11, 13, 16 and 42 are allowable. Applicants also gratefully acknowledge the Examiner's indication that the prior art cited on the IDS has been considered, but that none of the references are applied against the now claimed subject matter.

New Claims 44-53 have been added in order to more fully define preferred embodiments of the invention. All of the added claims are fully supported in the Specification. For example, Claims 44 and 45 find support on page 10, formula VI, page 40, and Table 7 on page 102. Claims 46-49 find support on pages 16-18, and in Examples 75-80. New Claim 50 finds support on pages 21 (lines 19-30), and on pages 41 and 97. New Claims 51-53 find support on page 21. No new matter is added by the added claims, and entry thereof is courteously requested.

In summary, Applicants have amended the Specification to correct several typographical errors, and to ensure full compliance with all limitations of generic formula I. Applicants have amended the claims so that they are drawn to the elected invention. Applicants have established that the present Specification provides a full, clear and concise description of how to make and how to use the claimed invention, and also that the claimed compounds have a believable utility, and one that is well recognized in the medical art. Applicants have established that the instant claims are not subject to rejection under 35 U.S.C. 102, because the cited Harris patent is not available as a reference since Applicants' application was filed well before the effective date of Harris. Applicants submit that the application is in condition for allowance, and courteously

request the Examiner to withdraw all rejections and objections and to pass the case to issue.

Respectfully submitted,

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Attachments - Amended claims - Version with markings to show changes made
Abstract
Current Opinion in Oncologic, Endocrine & Metabolic Investigational Drugs 2000, 2(1):40-59
J. Med. Chem., 2000, Vol. 43, No. 1, pp 1-18
Cancer Research, Vol. 60, No. 14, 2000, pp 3683-3987

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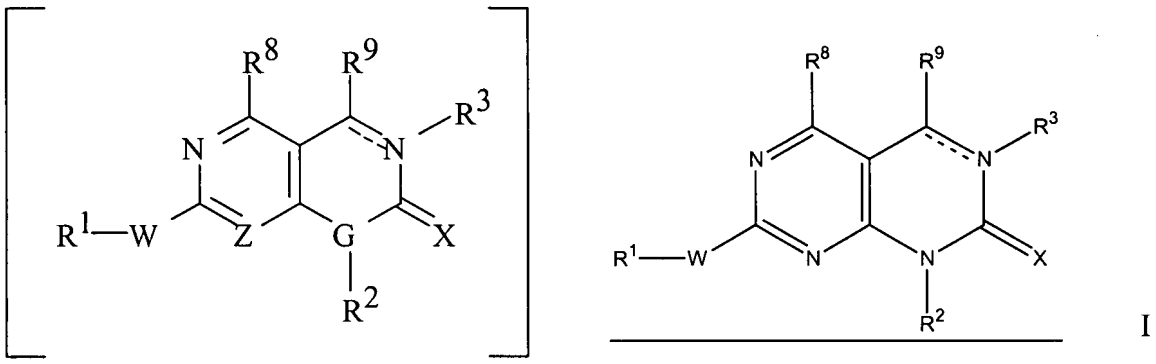
VERSION WITH MARKINGS TO SHOW CHANGES MADE

Claim 1 (amended). A compound of Formula I



I

and the pharmaceutically acceptable salts thereof,

wherein:

the dotted line represents an optional double bond;

[Z is N or CH;

G is N or CH;]

W is NH, S, SO, or SO_2;

X is either O, S, or NR^{10};

R^1, R^2, and R^{10} are independently selected from the group consisting of H, $(CH_2)_nAr$, COR^4, $(CH_2)_n$heteroaryl, $(CH_2)_n$heterocyclyl, C_1-C_{10} alkyl, C_3-C_{10} cycloalkyl, C_2-C_{10} alkenyl, and C_2-C_{10} alkynyl, wherein n is 0, 1, 2, or 3, and the $(CH_2)_nAr$, $(CH_2)_n$heteroaryl, alkyl, cycloalkyl, alkenyl, and alkynyl groups are optionally substituted by up to 5 groups selected from NR^4R^5, [$N(O)R^4R^5$, $NR^4R^5R^6Y$,] $\underline{N^+(O)R^4R^5}$, $\underline{N^+R^4R^5R^6Y^-}$, alkyl, phenyl, substituted phenyl, $(CH_2)_n$heteroaryl, hydroxy, alkoxy, phenoxy, thiol, thioalkyl, halo, COR^4, CO_2R^4, $CONR^4R^5$, $SO_2NR^4R^5$, SO_3R^4, PO_3R^4, aldehyde, nitrile, nitro,

heteroaryloxy, $T(CH_2)_mQR^4$, $T(CH_2)_mC(OR^5)H-(CH_2)_mQR^4$,

$C(O)T(CH_2)_mQR^4$, $NHC(O)T(CH_2)_mQR^4$, $T(CH_2)_mC(O)NR^4NR^5$, or $T(CH_2)_mCO_2R^4$ wherein each m is independently 1-6, T is O, S, NR^4, [$N(O)R^4$, NR^4R^6Y,] $\underline{N^+(O)R^4,\ N^+R^4R^6Y^-,}$ or CR^4R^5, and Q is O, S, NR^5, [$N(O)R^5$, or NR^5R^6Y] $\underline{N^+(O)R^5,\ or\ N^+R^5R^6Y^-}$;

when the dotted line is present, R^3 is absent;

otherwise R^3 has the meanings of R^2, wherein R^2 is as defined above, as well as OH, NR^4R^5, $COOR^4$, OR^4, $CONR^4R^5$, $SO_2NR^4R^5$, SO_3R^4, PO_3R^4,

$T(CH_2)_mQR^4$, $T(CH_2)_mC(OR^5)H-(CH_2)_mQR^4$,

wherein T and Q are as defined above;

R^4 and R^5 are each independently selected from the group consisting of hydrogen, C_1-C_6 alkyl, substituted alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, $N(C_1$-C_6alkyl$)_{1\ or\ 2}$, $(CH_2)_nAr$, C_3-C_{10} cycloalkyl, heterocyclyl, and heteroaryl, or R^4 and R^5 together with the nitrogen to which they are attached optionally form a ring having 3 to 7 carbon atoms and said ring optionally contains 1, 2, or 3 heteroatoms selected from the group consisting of nitrogen, substituted nitrogen, oxygen, and sulfur;

when R^4 and R^5 together with the nitrogen to which they are attached form a ring, the said ring is optionally substituted by 1 to 3 groups selected from OH, OR^4, NR^4R^5, $(CH_2)_mOR^4$, $(CH_2)_mNR^4R^5$, $T\text{-}(CH_2)_mQR_4$, $CO\text{-}T\text{-}(CH_2)_mQR^4$, $NH(CO)T(CH_2)_mQR^4$, $T\text{-}(CH_2)_mCO_2R^4$, or $T(CH_2)_mCONR^4R^5$.

R^6 is alkyl;

R^8 and R^9 independently are H, C_1-C_3 alkyl, NR^4R^5, [$N(O)R^4R^5$, $NR^4R^5R^6Y$,] $\underline{N^+(O)R^4R^5}$, $\underline{N^+R^4R^5R^6Y^-}$, hydroxy, alkoxy, thiol, thioalkyl, halo, COR^4, CO_2R^4, $CONR^4R^5$, $SO_2NR^4R^5$, SO_3R^4, PO_3R^4, CHO, CN, or NO_2;

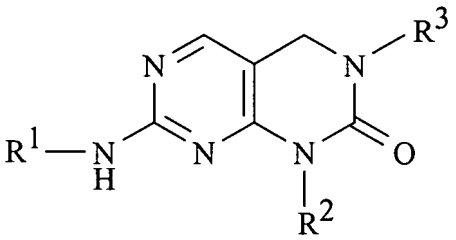
when the dotted line is absent, R^9 is additionally [carbonyl, thiocarbonyl, imine and substituted imine, oxime and oxime ether,] oxo,

S NH NR^4 NOH NOR^4
‖ ‖ ‖ ‖ ‖
C- R^4, CH, CH , CH , C , and

Y is a halo counter-ion.

Claim 2 (amended). A compound of Claim 1 wherein [Z and G both are N,] W is NH, and R^8, and R^9 both are hydrogen.

Claim 7 (amended). A compound of Claim 2 having the formula



[.]

wherein:

R^1, R^2 and R^3 independently are hydrogen, C_1-C_{10} alkyl, $(CH_2)_nAr$, $(CH_2)_n$heteroaryl, C_3-C_{10} cycloalkyl, $(CH_2)_n$ C_3-C_{10} cycloalkyl, or $(CH_2)_n$heteroaryl,

wherein n is 0, 1, 2 or 3, and the $(CH_2)_nAr$, $(CH_2)_n$heteroaryl, alkyl, cycloalkyl and heterocyclyl groups are optionally substituted by up to 5 groups

selected from NR^4R^5, $N^+(O)R^4R^5$, $N^+R^4R^5R^6Y^-$, alkyl, phenyl, substituted phenyl, $(CH_2)_n$heteroaryl, hydroxy, alkoxy, phenoxy, thiol, thioalkyl, halo, COR^4, CO_2R^4, $CONR^4R^5$, $SO_2NR^4R^5$, SO_3R^4, PO_3R^4, aldehyde, nitrile, nitro, heteroaryloxy, $T(CH_2)_mQR^4$,

$$T(CH_2)_mC(OR^5)(H)-(CH_2)_mQR^4,\ C(O)T(CH_2)_mQR^4,\ NHC(O)T(CH_2)_mQR^4,$$

$T(CH_2)_mC(O)NR^4NR^5$, or $T(CH_2)_mCO_2R^4$ wherein each m is independently 1-6, T is O, S, NR^4, $N^+(O)R^4$, $N^+R^4R^6Y^-$, or CR^4R^5, and Q is O, S, NR^5, $N^+(O)R^5$, or $N^+R^5R^6Y^-$;

when the dotted line is present, R^3 is absent;

otherwise R^3 has the meanings of R^2, wherein R^2 is as defined above, as well as OH, NR^4R^5, $COOR^4$, OR^4, $CONR^4R^5$, $SO_2NR^4R^5$, SO_3R^4, PO_3R^4,

$$T(CH_2)_mQR^4,\ T(CH_2)_mC(OR^5)(H)-(CH_2)_mQR^4,$$

wherein T and Q are as defined above;

R^4 and R^5 are each independently selected from the group consisting of hydrogen, C_1-C_6 alkyl, substituted alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, $N(C_1$-C_6alkyl$)_{1\text{ or }2}$, $(CH_2)_nAr$, C_3-C_{10} cycloalkyl, heterocyclyl, and heteroaryl, or R^4 and R^5 together with the nitrogen to which they are attached optionally form a ring having 3 to 7 carbon atoms and said ring optionally contains 1, 2, or 3 heteroatoms selected from the group consisting of nitrogen, substituted nitrogen, oxygen, and sulfur;

when R^4 and R^5 together with the nitrogen to which they are attached form a ring, the said ring is optionally substituted by 1 to 3 groups selected from

OH, OR^4, NR^4R^5, $(CH_2)_mOR^4$, $(CH_2)_mNR^4R^5$, $T\text{-}(CH_2)_mQR_4$, $CO\text{-}T\text{-}(CH_2)_mQR^4$, $NH(CO)T(CH_2)_mQR^4$, $T\text{-}(CH_2)_mCO_2R^4$, or $T(CH_2)_mCONR^4R^5$;

R^6 is alkyl;

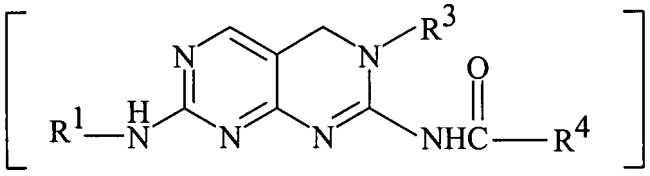
R^8 and R^9 independently are H, C_1-C_3 alkyl, NR^4R^5, $N^+(O)R^4R^5$, $N^+R^4R^5R^6Y^-$, hydroxy, alkoxy, thiol, thioalkyl, halo, COR^4, CO_2R^4, $CONR^4R^5$, $SO_2NR^4R^5$, SO_3R^4, PO_3R^4, CHO, CN, or NO_2;

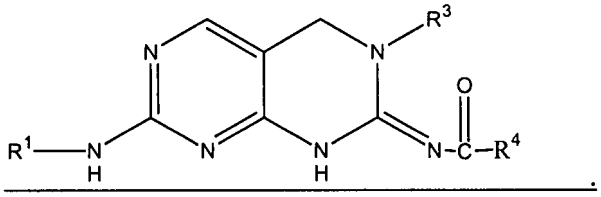
when the dotted line is absent, R^9 is additionally oxo,

S	NH	NR^4	NOH	NOR^4	
C- R^4,	CH,	CN,	CH,	C,	and

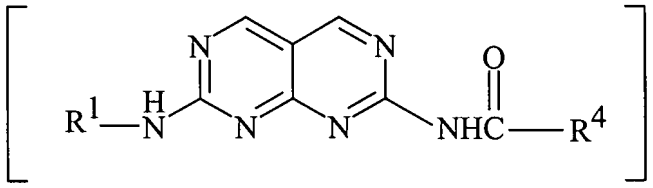
Y is a halo counter-ion.

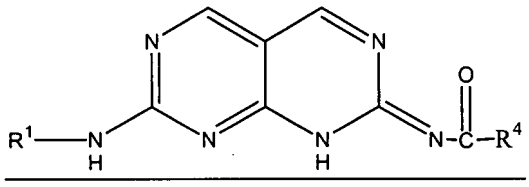
Claim 10 (amended). A compound of Claim 2 having the formula





Claim 12 (amended). A compound of Claim 2 having the formula





Claim 17 (cancelled).

Claim 18 (cancelled).

Claim 19 (cancelled).

Claim 20 (cancelled).

Claim 21 (cancelled).

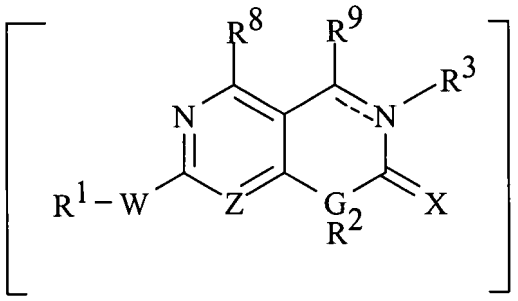
Claim 22 (cancelled).

Claim 23 (cancelled).

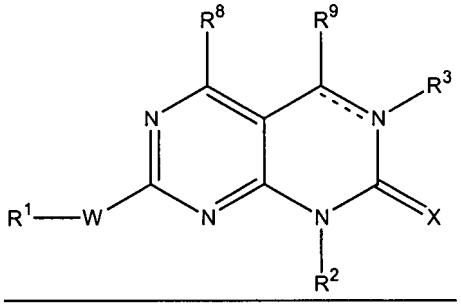
Claim 24 (cancelled).

Claim 25 (cancelled).

Claim 26 (amended). A method of inhibiting a cyclin-dependent kinase comprising contacting the cyclin-dependent kinase with a compound of Formula I



I



and the pharmaceutically acceptable salts thereof,

wherein:

the dotted line represents an optional double bond;

W is NH, S, SO, or SO_2;

X is either O, S, or NR^{10};

R^1, R^2, and R^{10} are independently selected from the group consisting of H, $(CH_2)_nAr$, COR^4, $(CH_2)_n$heteroaryl, $(CH_2)_n$heterocyclyl, C_1-C_{10} alkyl, C_3-C_{10} cycloalkyl, C_2-C_{10} alkenyl, and C_2-C_{10} alkynyl, wherein n is 0, 1, 2, or 3, and the $(CH_2)_nAr$, $(CH_2)_n$heteroaryl, alkyl, cycloalkyl, alkenyl, and alkynyl groups are optionally substituted by up to 5 groups selected from NR^4R^5, [$N(O)R^4R^5$, $NR^4R^5R^6Y$,] $N^+(O)R^4R^5$, $N^+R^4R^5R^6Y^-$, alkyl, phenyl, substituted phenyl, $(CH_2)_n$heteroaryl, hydroxy, alkoxy, phenoxy, thiol, thioalkyl, halo, COR^4, CO_2R^4, $CONR^4R^5$, $SO_2NR^4R^5$, SO_3R^4, PO_3R^4, aldehyde, nitrile, nitro,

heteroaryloxy, $T(CH_2)_mQR^4$, $T(CH_2)_mC(OR^5)H-(CH_2)_mQR^4$,

$C(O)T(CH_2)_mQR^4$, $NHC(O)T(CH_2)_mQR^4$, $T(CH_2)_mC(O)NR^4NR^5$, or $T(CH_2)_mCO_2R^4$ wherein each m is independently 1-6, T is O, S, NR^4, [$N(O)R^4$, NR^4R^6Y,] $\underline{N^+(O)R^4, N^+R^4R^6Y^-,}$ or CR^4R^5, and Q is O, S, NR^5, [$N(O)R^5$, or NR^5R^6Y] $\underline{N^+(O)R^5, \text{ or } N^+R^5R^6Y^-}$;

when the dotted line is present, R^3 is absent;

otherwise R^3 has the meanings of R^2, wherein R^2 is as defined above, as well as OH, NR^4R^5, $COOR^4$, OR^4, $CONR^4R^5$, $SO_2NR^4R^5$, SO_3R^4, PO_3R^4,

$T(CH_2)_mQR^4$, $T(CH_2)_mC(OR^5)H-(CH_2)_mQR^4$,

wherein T and Q are as defined above;

R^4 and R^5 are each independently selected from the group consisting of hydrogen, C_1-C_6 alkyl, substituted alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, $N(C_1$-C_6alkyl$)_{1 \text{ or } 2}$, $(CH_2)_nAr$, C_3-C_{10} cycloalkyl, heterocyclyl, and heteroaryl, or R^4 and R^5 together with the nitrogen to which they are attached optionally form a ring having 3 to 7 carbon atoms and said ring optionally contains 1, 2, or 3 heteroatoms selected from the group consisting of nitrogen, substituted nitrogen, oxygen, and sulfur;

when R^4 and R^5 together with the nitrogen to which they are attached form a ring, the said ring is optionally substituted by 1 to 3 groups selected from OH, OR^4, NR^4R^5, $(CH_2)_mOR^4$, $(CH_2)_mNR^4R^5$, $T\text{-}(CH_2)_mQR_4$, $CO\text{-}T\text{-}(CH_2)_mQR^4$, $NH(CO)T(CH_2)_mQR^4$, $T\text{-}(CH_2)_mCO_2R^4$, or $T(CH_2)_mCONR^4R^5$; [.]

R^6 is alkyl;

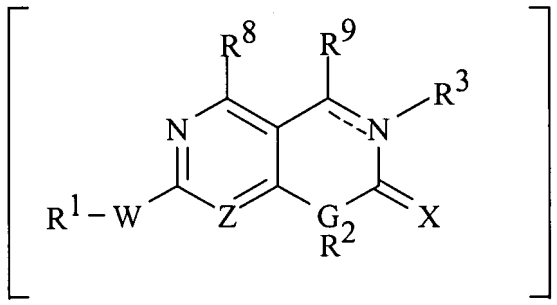
R^8 and R^9 independently are H, C_1-C_3 alkyl, NR^4R^5, [$N(O)R^4R^5$, $NR^4R^5R^6Y$,] $\underline{N^+(O)R^4R^5, N^+R^4R^5R^6Y^-}$, hydroxy, alkoxy, thiol, thioalkyl, halo, COR^4, CO_2R^4, $CONR^4R^5$, $SO_2NR^4R^5$, SO_3R^4, PO_3R^4, CHO, CN, or NO_2;

when the dotted line is absent, R^9 is additionally [carbonyl, thiocarbonyl, imine and substituted imine, oxime and oxime ether,] oxo,

S	NH	NR^4	NOH	NOR^4	
‖	‖	‖	‖	‖	
C- R^4,	CH,	CH ,	CH ,	C	, and

Y is a halo counter-ion.

Claim 30 (amended). A method of inhibiting a growth factor-mediated tyrosine kinase comprising contacting said growth factor-mediated kinase with a compound of Formula I



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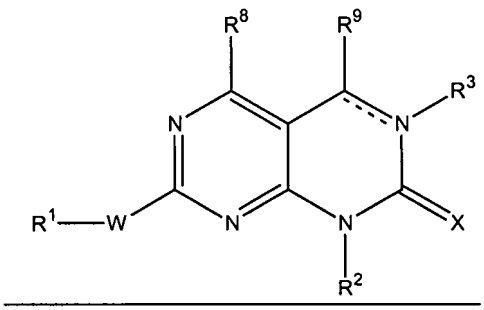


and the pharmaceutically acceptable salts thereof,

wherein:

the dotted line represents an optional double bond;

[Z is N or CH;

G is N or CH;]

W is NH, S, SO, or SO_2;

X is either O, S, or NR^{10};

R^1, R^2, and R^{10} are independently selected from the group consisting of H, $(CH_2)_nAr$, COR^4, $(CH_2)_n$heteroaryl, $(CH_2)_n$heterocyclyl, C_1-C_{10} alkyl, C_3-C_{10} cycloalkyl, C_2-C_{10} alkenyl, and C_2-C_{10} alkynyl, wherein n is 0, 1, 2, or 3, and the $(CH_2)_nAr$, $(CH_2)_n$heteroaryl, alkyl, cycloalkyl, alkenyl, and alkynyl groups are optionally substituted by up to 5 groups selected from NR^4R^5, [$N(O)R^4R^5$, $NR^4R^5R^6Y$,] $\underline{N^+(O)R^4R^5, N^+R^4R^5R^6Y^-,}$ alkyl, phenyl, substituted phenyl, $(CH_2)_n$heteroaryl, hydroxy, alkoxy, phenoxy, thiol, thioalkyl, halo, COR^4, CO_2R^4, $CONR^4R^5$, $SO_2NR^4R^5$, SO_3R^4, PO_3R^4, aldehyde, nitrile, nitro,

$$\text{heteroaryloxy, } T(CH_2)_mQR^4, \ T(CH_2)_mC(OR^5)H\text{-}(CH_2)_mQR^4,$$

$C(O)T(CH_2)_mQR^4$, $NHC(O)T(CH_2)_mQR^4$, $T(CH_2)_mC(O)NR^4NR^5$, or $T(CH_2)_mCO_2R^4$ wherein each m is independently 1-6, T is O, S, NR^4, [$N(O)R^4$, NR^4R^6Y,] $\underline{N^+(O)R^4, N^+R^4R^6Y^-,}$ or CR^4R^5, and Q is O, S, NR^5, [$N(O)R^5$, or NR^5R^6Y] $\underline{N^+(O)R^5, \text{ or } N^+R^5R^6Y^-}$;

when the dotted line is present, R^3 is absent;

otherwise R^3 has the meanings of R^2, wherein R^2 is as defined above, as well as OH, NR^4R^5, $COOR^4$, OR^4, $CONR^4R^5$, $SO_2NR^4R^5$, SO_3R^4, PO_3R^4,

$$T(CH_2)_mQR^4,\ T(CH_2)_mC(OR^5)H\text{-}(CH_2)_mQR^4,$$

wherein T and Q are as defined above;

R^4 and R^5 are each independently selected from the group consisting of hydrogen, C_1-C_6 alkyl, substituted alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, $N(C_1\text{-}C_6alkyl)_{1 \text{ or } 2}$, $(CH_2)_nAr$, C_3-C_{10} cycloalkyl, heterocyclyl, and heteroaryl, or R^4 and R^5 together with the nitrogen to which they are attached optionally form a ring having 3 to 7 carbon atoms and said ring optionally contains 1, 2, or 3 heteroatoms selected from the group consisting of nitrogen, substituted nitrogen, oxygen, and sulfur;

when R^4 and R^5 together with the nitrogen to which they are attached form a ring, the said ring is optionally substituted by 1 to 3 groups selected from OH, OR^4, NR^4R^5, $(CH_2)_mOR^4$, $(CH_2)_mNR^4R^5$, $T\text{-}(CH_2)_mQR_4$, $CO\text{-}T\text{-}(CH_2)_mQR^4$, $NH(CO)T(CH_2)_mQR^4$, $T\text{-}(CH_2)_mCO_2R^4$, or $T(CH_2)_mCONR^4R^5$; [.]

R^6 is alkyl;

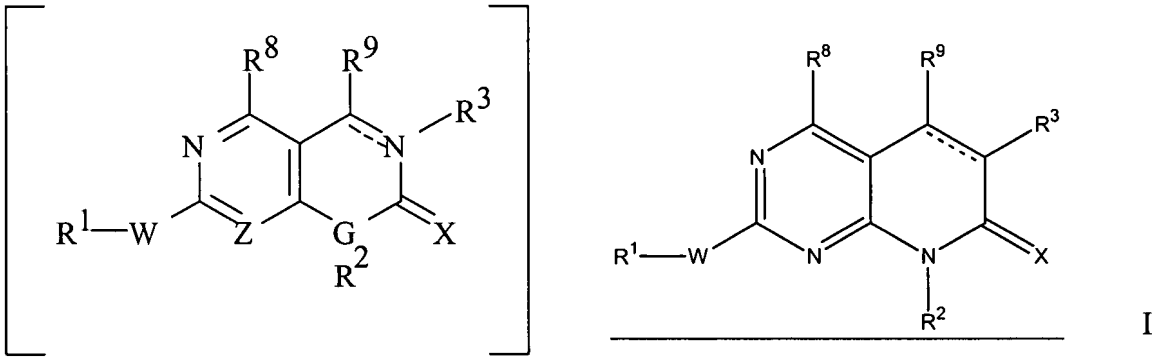
R^8 and R^9 independently are H, C_1-C_3 alkyl, NR^4R^5, [$N(O)R^4R^5$, $NR^4R^5R^6Y$,] $N^+(O)R^4R^5$, $N^+R^4R^5R^6Y^-$, hydroxy, alkoxy, thiol, thioalkyl, halo, COR^4, CO_2R^4, $CONR^4R^5$, $SO_2NR^4R^5$, SO_3R^4, PO_3R^4, CHO, CN, or NO_2;

when the dotted line is absent, R^9 is additionally [carbonyl, thiocarbonyl, imine and substituted imine, oxime and oxime ether,] oxo,

$$C(=S)\text{-}R^4,\ CH(=NH),\ CH(=NR^4),\ CH(=NOH),\ C(=NOR^4),$$ and

Y is a halo counter-ion.

Claim 34 (amended). A method of inhibiting a non-receptor tyrosine kinase comprising contacting said non-receptor tyrosine kinase with a compound of Formula I



I

and the pharmaceutically acceptable salts thereof,

wherein:

the dotted line represents an optional double bond;

[Z is N or CH;

G is N or CH;]

W is NH, S, SO, or SO_2;

X is either O, S, or NR^{10};

R^1, R^2, and R^{10} are independently selected from the group consisting of H, $(CH_2)_nAr$, COR^4, $(CH_2)_n$heteroaryl, $(CH_2)_n$heterocyclyl, C_1-C_{10} alkyl, C_3-C_{10} cycloalkyl, C_2-C_{10} alkenyl, and C_2-C_{10} alkynyl, wherein n is 0, 1, 2, or 3, and the $(CH_2)_nAr$, $(CH_2)_n$heteroaryl, alkyl, cycloalkyl, alkenyl, and alkynyl groups are optionally substituted by up to 5 groups selected from NR^4R^5, [$N(O)R^4R^5$, $NR^4R^5R^6Y$] $\underline{N^+(O)R^4R^5, N^+R^4R^5R^6Y^-}$, alkyl, phenyl, substituted phenyl, $(CH_2)_n$heteroaryl, hydroxy, alkoxy, phenoxy, thiol, thioalkyl, halo, COR^4, CO_2R^4, $CONR^4R^5$, $SO_2NR^4R^5$, SO_3R^4, PO_3R^4, aldehyde, nitrile, nitro,

heteroaryloxy, $T(CH_2)_mQR^4$, $T(CH_2)_mC(OR^5)H-(CH_2)_mQR^4$,

$C(O)T(CH_2)_mQR^4$, $NHC(O)T(CH_2)_mQR^4$, $T(CH_2)_mC(O)NR^4NR^5$, or $T(CH_2)_mCO_2R^4$ wherein each m is independently 1-6, T is O, S, NR^4, [$N(O)R^4$, NR^4R^6Y] $\underline{N^+(O)R^4, N^+R^4R^6Y^-}$, or CR^4R^5, and Q is O, S, NR^5, $N(O)R^5$, or NR^5R^6Y;

when the dotted line is present, R^3 is absent;

otherwise R^3 has the meanings of R^2, wherein R^2 is as defined above, as well as OH, NR^4R^5, $COOR^4$, OR^4, $CONR^4R^5$, $SO_2NR^4R^5$, SO_3R^4, PO_3R^4,

$T(CH_2)_mQR^4$, $T(CH_2)_mC(OR^5)H-(CH_2)_mQR^4$,

wherein T and Q are as defined above;

R^4 and R^5 are each independently selected from the group consisting of hydrogen, C_1-C_6 alkyl, substituted alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, $N(C_1$-C_6alkyl$)_{1 \text{ or } 2}$, $(CH_2)_nAr$, C_3-C_{10} cycloalkyl, heterocyclyl, and heteroaryl, or R^4 and R^5 together with the nitrogen to which they are attached optionally form a ring having 3 to 7 carbon atoms and said ring optionally contains 1, 2, or 3 heteroatoms selected from the group consisting of nitrogen, substituted nitrogen, oxygen, and sulfur;

when R^4 and R^5 together with the nitrogen to which they are attached form a ring, the said ring is optionally substituted by 1 to 3 groups selected from OH, OR^4, NR^4R^5, $(CH_2)_mOR^4$, $(CH_2)_mNR^4R^5$, T-$(CH_2)_mQR_4$,

$CO\text{-}T\text{-}(CH_2)_mQR^4$, $NH(CO)T(CH_2)_mQR^4$, $T\text{-}(CH_2)_mCO_2R^4$, or $T(CH_2)_mCONR^4R^5$.

R^6 is alkyl;

R^8 and R^9 independently are H, C_1-C_3 alkyl, NR^4R^5, [$N(O)R^4R^5$, $NR^4R^5R^6Y$] $\underline{N^+(O)R^4R^5,\ N^+R^4R^5R^6Y^-}$, hydroxy, alkoxy, thiol, thioalkyl, halo, COR^4, CO_2R^4, $CONR^4R^5$, $SO_2NR^4R^5$, SO_3R^4, PO_3R^4, CHO, CN, or NO_2;

when the dotted line is absent, R^9 is additionally [carbonyl, thiocarbonyl, imine and substituted imine, oxime and oxime ether,] oxo,

$\underline{\overset{S}{\|}\text{-}R^4,\ \overset{NH}{\|}\text{,}\ \overset{NR^4}{\|}\text{,}\ \overset{NOH}{\|}\text{,}\ \overset{NOR^4}{\|}}$ and

Y is a halo counter-ion.

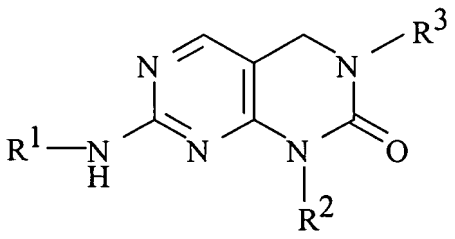
Claim 37 (amended). A method of treating a subject suffering from [diseases caused by] vascular smooth muscle cell proliferation comprising administering to said subject a therapeutically effective amount of a compound of Claim 1.

Claim 38 (cancelled).

Claim 40 (cancelled).

Please add new Claims 44-53:

Claim 44 (new). A compound of the formula



wherein:

R^1 is C_1-C_{10} alkyl or $(CH_2)_nAr$;

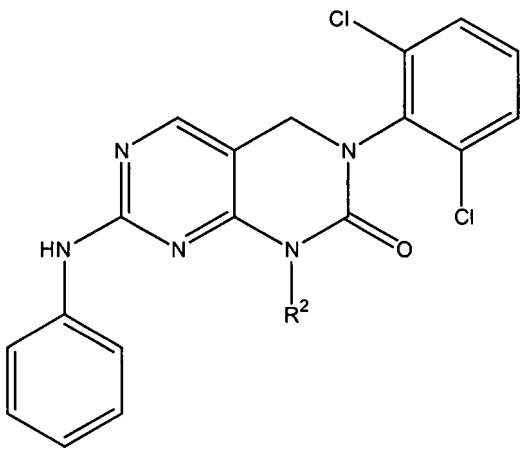
R^2 is H, C_1-C_{10} alkyl, or $(CH_2)_nAr$; and

R^3 is Ar,

wherein n is 0, 1, 2 or 3;

Ar is phenyl or phenyl substituted with one or two groups selected from halo, alkyl, or substituted alkyl; or a pharmaceutically acceptable salt thereof.

Claim 45 (new). A compound of the formula



wherein R^2 is $(CH_2)_nAr$, n is 0, 1, 2 or 3, and Ar is phenyl or phenyl substituted by a 2-aminoethyl group,
or a pharmaceutically acceptable salt thereof.

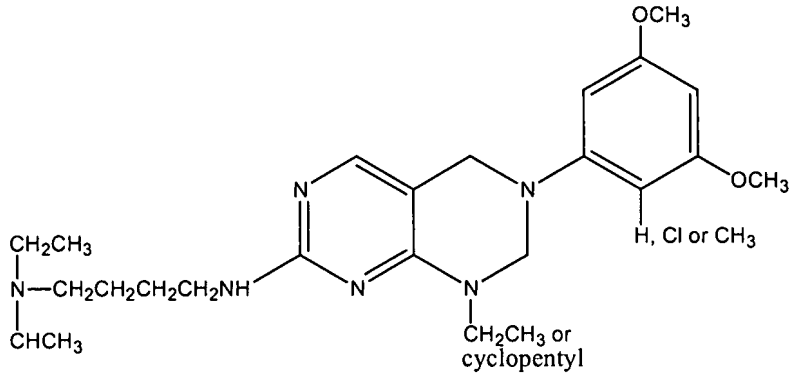
Claim 46 (new). A pharmaceutical formulation comprising a compound of Claim 3 in combination with a pharmaceutically acceptable carrier, diluent or excipient.

Claim 47 (new). A pharmaceutical formulation comprising a compound of Claim 7 in combination with a pharmaceutically acceptable carrier, diluent or excipient.

Claim 48 (new). A pharmaceutical formulation comprising a compound of Claim 44 in combination with a pharmaceutically acceptable carrier, diluent or excipient.

Claim 49 (new). A pharmaceutical formulation comprising a compound of Claim 45 in combination with a pharmaceutically acceptable carrier, diluent or excipient.

Claim 50 (new). A compound of the formula



or a pharmaceutically acceptable salt thereof.

Claim 51 (new). The compound 7-(4-diethylamino-butylamino)-3-(2-chloro-3,5-dimethoxy-phenyl)-1-ethyl-3,4-dihydro-pyrimido[4,5-*d*]pyrimidine-2(1*H*)-one.

Claim 52 (new). The compound 7-(4-diethylamino-butylamino)-3-(2-methyl-3,5-dimethoxy-phenyl)-1-ethyl-3,4-dihydro-pyrimido[4,5-*d*]pyrimidine-2(1*H*)-one.

Claim 53 (new). The compound 7-(4-diethylamino-butylamino)-3-(3,5-dimethoxy-phenyl)-1-cyclopentyl-3,4-dihydro-pyrimido[4,5-*d*]pyrimidine-2(1*H*)-one.

Inhibitors of cyclin-dependent kinases as therapeutic agents for the treatment of cancer

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Introduction

The regulation of cell cycle progression and cell division is an enormously complex process involving numerous regulatory processes, including protein phosphorylation/dephosphorylation, transcriptional and translational controls, protein degradation and the presence of a myriad of naturally occurring protein activators and inhibitors [1-4]. All of these events represent a complex set of positive and negative signals, checkpoints and feedback controls, all governed to maintain the fidelity of DNA synthesis, the timely occurrence of mitosis and the formation of new daughter cells in which the integrity of the genome remains intact. Many of these processes focus on the timely activation and inactivation of the cyclin-dependent kinases (CDKs), which have been identified as key mediators of cell cycle progression [5-8]. Because of the key role that these enzymes play in the progression through the mammalian cell cycle, CDKs have been considered attractive chemotherapeutic targets for most proliferative diseases [7,9-11]. In particular, the rationale to inhibit certain CDKs as a specific approach to cancer chemotherapy has become progressively stronger as research in this area continues to identify tumor-related abnormalities in the cell cycle that appear to contribute to the development and progression of cancer.

Although the complex and multi-component mechanisms that regulate CDKs provide varied approaches to modulating their catalytic activity, there has been considerable effort to identify low molecular weight molecules that directly inhibit their catalytic activity. The drug-discovery efforts and properties of these earlier inhibitors have been addressed in a number of excellent review articles [12-17]. As has generally been the case for inhibitors of other kinase targets, the development of CDK inhibitors has been an evolutionary process, beginning with molecules that usually originated as natural products but lacked potency and specificity. Within the last two years, however, the patent literature has exploded with new synthetic molecules that have been identified from screening large chemical and combinatorial libraries. Novel structural classes of compounds obtained from these libraries have been optimized for potency and specificity using information from structure-activity relationships, CDK-inhibitor co-crystal structures, molecular modeling and computer-aided drug design.

This review will present the current status of cell cycle regulation, discuss the existing evidence that provides a rationale for the inhibition of cyclin-dependent kinases as an approach to cancer chemotherapy and present an overview of the chemical structures and properties of existing cyclin-dependent kinase inhibitors. These include the most recently disclosed molecules in the patent literature.

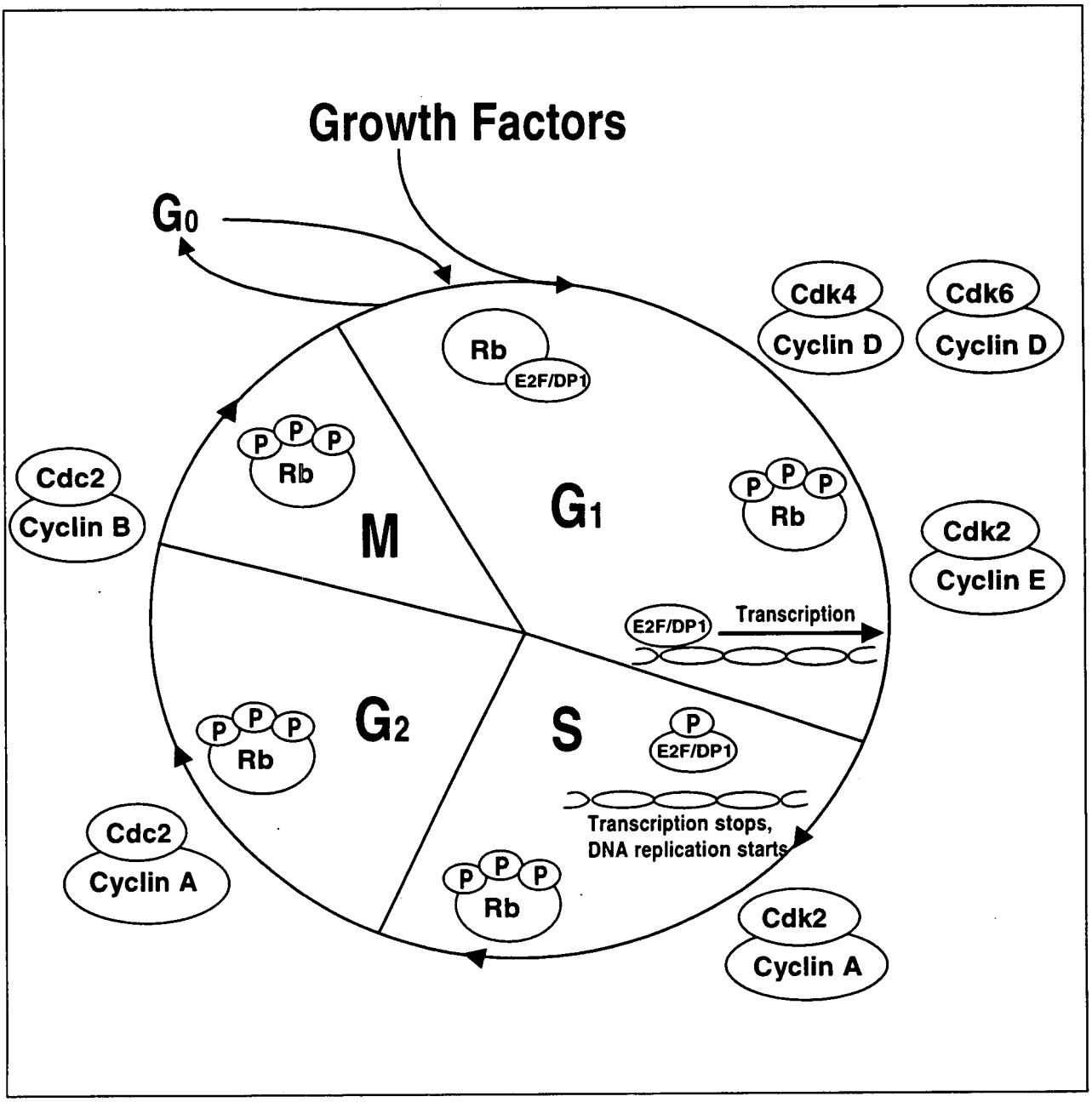
Fundamentals of cell cycle regulation and progression

The cell division cycle is the process by which a cell grows, replicates its DNA and then divides to give two daughter cells. It can be divided into four sequential phases, G1, S, G2 and M [18]. The first of these, G1 (also known as Gap1), is the period before DNA replication when a cell is growing and responsive to extracellular stimuli. This is followed by S phase, when a cell replicates its DNA, and then a second Gap, G2, when the cell prepares for entry into mitosis. Mitosis occurs when the replicated DNA is segregated and cell division occurs to give two genetically identical daughter cells. Finally, there is a fifth state known as G0 (or quiescence), into which cells may reversibly exit from the cell cycle but remain metabolically active.

Looking at the phases that make up the cell cycle, one may ask, how does a cell ensure that it has completed one phase of the cell cycle before moving onto the next? Secondly, how does a cell transiently stop in the cell cycle when one phase has not been accurately completed, such as when it suffers DNA damage that needs to be repaired? These questions have been answered with the identification of the machinery that regulates the cell cycle clock. At the heart of this machinery is the cyclin-dependent kinase family (CDK) of serine/threonine kinases. For full activity, these kinases must be phosphorylated on a specific threonine residue (Thr161 for cdc2 which is also known as cdk1 in some literature) and form a complex with a member of the cyclin family of regulatory subunits [19,20]. These active CDK/cyclin complexes then regulate cell cycle progression by phosphorylation of protein substrates whose activities are required at specific phases of the cell cycle. Different CDK/cyclin complexes are required for cell cycle progression at different phases of the cell cycle and phosphorylate an assortment of substrates [20]. For

example, when a quiescent cell is stimulated by growth factors to leave the G0 state and enter the cell cycle, it responds by expressing one or more of the three D-type cyclins (D1, D2, D3) [21]. These then associate with cdk4 and cdk6, the CDK/cyclin complexes become activated and phosphorylate the protein product of the tumor suppressor gene *Rb* (Rb) (see Figure 1). In mid to late G1, the E-type cyclins E1 and E2 are expressed and associate with cdk2 to give CDK/cyclin complexes, which also phosphorylate Rb. It is this sequential phosphorylation of Rb by cyclin D-dependent kinases followed by cdk2/cyclin E that blocks its growth inhibitory functions. For example, in early G1, Rb is hypophosphorylated and tightly binds and represses the activity of E2F1/DP1 (also known simply as E2F), a transcription factor complex which is required for the expression of genes necessary for S phase. As G1 progresses and Rb becomes phosphorylated, its interaction with E2F is disrupted and E2F-dependent transcription occurs (see Figure 1). This allows the cell to pass a point in G1 known as the restriction point (R) after which the cell no longer needs mitogenic stimulation to complete one cell cycle. So far only the Rb family of related proteins appear to be substrates for cyclin D-dependent kinases, whilst cdk2/cyclin E complexes have been shown to phosphorylate several distinct substrates, at least in vitro [22-24]. As cells leave G1 and enter S phase, the A-type cyclins are expressed and form complexes with cdk2 to give a kinase activity that can phosphorylate the transcription factors B-Myb and E2F, and cdc6, a factor required for the initiation of DNA replication [25-28]. As S phase progresses into G2, cdc2/cyclin A complexes can also be found [29,30]. In late G2, however, the predominant complex is cdc2/cyclin B. The Cdc2/cyclin B

Figure 1. The mammalian cell cycle.



complex was originally identified as M phase-promoting factor (MPF), having activity required to promote entry of cells into M phase [31]. Cdc2/cyclin B complexes have been shown to phosphorylate a number of proteins both *in vitro* and *in vivo*, many of which are involved in the early events of mitosis [32-34]. In summary therefore, it is the sequential activation of CDK/cyclin complexes and their ability to phosphorylate and thus regulate the actions of other proteins that allow a cell to progress from one completed phase of the cell cycle to the next, in the correct order. However, in order to discuss the role of CDK/cyclins in cell cycle checkpoints, it is first necessary to explore how these complexes are regulated.

Because specific CDK/cyclin complexes are required only at certain points in the cell cycle, their activities are tightly regulated. As mentioned earlier, full CDK activity requires association with a cyclin regulatory subunit, but also phosphorylation on a threonine residue (eg, Thr161 in the case of cdc2). An enzyme known as CAK (CDK Activating Kinase) carries out this threonine phosphorylation. CAK is itself a protein complex made up of a CDK (cdk7), a cyclin (cyclin H) and a third subunit p36/MAT1 [35]. Until recently it was believed that this was the only CAK in mammalian cells. However, a new report suggests that there may be an activity distinct from cdk7 that can carry out this function on cdk2 [36].

Negative regulation of CDK/cyclin activity is achieved in a number of ways. The first of these to be discovered was timed degradation of the cyclin subunit itself. This was subsequently found to be via ubiquitin-mediated proteolysis [37]. Phosphorylation can also play a negative regulatory role on CDKs. Cdc2 is phosphorylated on Tyr15 and Thr14 and these modifications on cdc2 appear to inhibit the activity of the complex [38]. Phosphorylation at these sites is carried out in mammalian cells by two kinases, Wee1, which predominantly phosphorylates cdc2 on Tyr15 and Myt1, which phosphorylates cdc2 on Thr14 [39,40]. This allows cdc2/cyclin B complexes formed during G2 to be inactive until dephosphorylation occurs at Thr14 and Tyr15 by the dual specificity phosphatase, cdc25C. Cdc2/cyclin B then becomes active and the cell is able to enter mitosis [41]. Most of the CDKs have threonine and tyrosine at the equivalent positions in their sequence. Two interesting exceptions are the cyclin D-associated kinases, cdk4 and cdk6, which lack a threonine equivalent to position 14 of cdc2. However, there is evidence that phosphorylation at the site equivalent to Tyr15 of cdc2 does indeed inhibit the function of both cdk4 and cdk6 in cells [42-44]. More recent studies indicate the existence of two families of CDK inhibitory proteins (CKIs), the CIP/KIP family comprising $p21^{CIP1/WAF1}$, $p27^{KIP1}$ and $p57^{KIP2}$ [45], and the INK4 family comprising $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$ [46]. These two families are structurally unrelated and, whilst the CIP/KIP family binds and inhibits a broad range of CDK/cyclin complexes *in vitro*, the INK4 family only binds and inhibits cdk4 and cdk6.

Another integral part of cell cycle control is governed by specific checkpoints. When a normal cell is subject to an external agent that causes DNA damage, it can transiently stop in any phase of the cell cycle in order to repair the damage and then resume cell cycle progression. Depending on the type and extent of the damage the cell also has the option to either enter quiescence or to undergo apoptosis. The ability of the cell to sense this damage and to transiently stop in the cell cycle is known as a checkpoint function [47, 48]. Such checkpoints have been identified for all phases of the progressing cell cycle and for at least two checkpoints, G1/S and G2/M, there is strong evidence of CDK involvement [47,48].

The G1 checkpoint appears to be dependent on the product p53 of the tumor suppressor gene, *p53*. This protein is sensitive to signals generated inside the cell in response to changes in metabolism or genomic integrity and, once the level of p53 in the cell is increased, it can invoke both cell cycle arrest and/or apoptosis [47-49]. It does this through transcriptional activation of promoters that contain binding sites for p53. One of these genes is the CKI $p21^{CIP1/WAF1}$, which can bind to CDKs and induce a cell cycle arrest. Another is the pro-apoptotic protein, Bax, which may be involved in the p53-dependent apoptotic response. Another factor in the G1 checkpoint may be the inhibitory phosphorylation of CDKs, since upon a UV-invoked G1 checkpoint, cdk4 function is inhibited by tyrosine phosphorylation [42,44]. Although the function of cdk2-associated kinase activity can also be inhibited by phosphorylation on residues equivalent to Thr14 and Tyr15 of cdc2, it appears that the activity of cdk2 at the G1 checkpoint is inhibited by $p21^{CIP1/WAF1}$ [50,51].

As mentioned earlier, normal cell entry into mitosis from G2 is controlled by the activity of cdc2/cyclin B. This is also true of the G2 DNA damage-induced checkpoint. Upon treatment of cells with genotoxic agents such as ionizing radiation or the chemotherapeutic, doxorubicin, transition of cells from G2 into mitosis is prevented by shutting off cdc2/cyclin B activity. This is accomplished by maintaining phosphorylation of cdc2 on Thr14 and Tyr15 [48], which is achieved by a series of events starting with activation of the phosphatidylinositol 3-kinase-like kinase, ATM [52]. Activation of ATM leads to phosphorylation and activation of two functionally- but not structurally-related kinases, Chk1 and Chk2. These kinases then phosphorylate the phosphatase, cdc25C, on a serine residue at position 216, which promotes the association of cdc25C with 14-3-3 proteins. The consequence of this action is that cdc25C is sequestered in the cytoplasm and thus cannot dephosphorylate cdc2/cyclin B complexes that are in the nucleus and inactive due to phosphorylation on Thr14 and Tyr15. Thus, cdc2 remains inactive and the cells arrest in G2. Recently it has become clear that this process determines how the G2 arrest is initiated; however, maintenance of this checkpoint requires a p53-dependent process [53-55]. As mentioned earlier, DNA damage activates p53 and this can then transcriptionally induce expression of the CKI $p21^{CIP1/WAF1}$, which can bind to and inhibit the activity of cdc2/cyclin B in the nucleus [53]. It also induces expression of the σ-form of 14-3-3, which does not bind to cdc25C [54]. 14-3-3 σ Instead appears to sequester cdc2/cyclin B complexes in the cytoplasm and thus away from the nucleus, where they are required. It has also been shown that immunoprecipitations of 14-3-3 σ contain cdc2 and Wee1 [54]. Thus, another function of 14-3-3 σ may be to facilitate interaction between cdc2 and Wee1 so that negative regulation of cdc2 activity by tyrosine phosphorylation is carried out. In conclusion, it is clear that both CDKs and their regulators play key roles in both the G1 and G2 damage-induced checkpoints.

Cancer, CDKs and the cell cycle

An inherent property of cancer is a cell division cycle that is no longer responsive to the appropriate regulatory signals. This is due to genetic alterations in the cell, many of which occur in the two key regulators of cell cycle progression, p53 and Rb. Indeed, more than 50% of all cancers have mutations in p53 [56,57]. However, there is mounting evidence that other members of the p53 and Rb regulatory pathways are also altered in human cancer.

Most human cancers contain mutations that affect members of the Rb pathway involved in regulation of G1 progression [58]. These include cdk4, the D-type cyclins, cdk2, cyclin E and the CKIs $p15^{INK4B}$, $p16^{INK4A}$, $p27^{KIP1}$, $p57^{KIP2}$, all of which could lead to inappropriate phosphorylation and hence inactivation of Rb [58-64]. These genetic changes occur through a number of different mechanisms, comprising chromosomal translocation or inversion (cyclin D1), amplification (cdk4, cdk6, cyclin D1, D2, D3), mutation leading to constitutive activity (cdk4), point mutation or deletion leading to inactivation ($p15^{INK4A}$, $P16^{INK4B}$, $p57^{KIP2}$) and proviral insertion (cyclin D2). There is also silencing of the promoter via methylation ($p16^{INK4A}$, $p27^{KIP1}$) and an increase ($p27^{KIP1}$) or decrease (cyclin D1, D3) in protein turnover, for which alterations at the gene level have yet to be identified. Whilst some of these mechanisms appear to be used by a number of cancer types to deregulate the Rb pathway, several are specific to one or perhaps two forms of cancer. For example, p27 expression is lower in more aggressive tumors of the breast, colon, gastric tract, lung and prostate, suggesting a fairly general mechanism of deregulation [65]. Interestingly, in colorectal cancer, low p27 expression is a result of an increase in protein turnover via ubiquitin-mediated proteolysis rather than genetic alteration of $p27^{KIP1}$, so in this case proteolytic degradation may be the target of genetic changes [66]. In contrast, the translocation (t11:14) which activates expression of cyclin D1 is fairly specific to mantle cell lymphoma and is found in 70% of these cases [67]. At the moment it is unclear why these preferences occur. It has also been noted that at least in certain forms of cancer, mutations of the Rb pathway are mutually exclusive. That is, if a tumor has a mutation in Rb, then it often does not appear to require a mutation in another member of the Rb pathway.

Finally, whilst it is fair to say that mutations of the Rb pathway are associated with deregulated proliferation they may also contribute to the transformed phenotype in another fashion. A recent report indicates that overexpression of cyclin E in both immortalized rat embryo fibroblasts and human breast epithelial cells results in chromosome instability [68]. Thus, downregulation of cdk2/cyclin E activity may be required as cells exit from G1 in order to maintain genomic stability.

Links with cancer appear to be less frequent or direct for other members of the cyclin and CDK families. For cyclin A, overexpression of the protein appears to correlate with poor prognosis in a number of cancers; however it is not clear whether this is a causative factor in tumor progression or just a marker of proliferation [69-71]. Reports of mutation, deletion or amplification of the gene appear to be rather rare. Interestingly, however, there have been reports of hepatitis B virus integration into an intron of the cyclin A gene in hepatocellular carcinoma, leading to a stabilized version of cyclin A, and a recent report suggesting that this form of cyclin A can contribute to cellular transformation [72-74]. With regard to cdc2/cyclin B, it is clear this activity plays a critical role both at the G2/M transition and the G2 checkpoint, but so far the genes encoding these proteins do not appear to be the target of genetic alteration in cancer. However, pathways that regulate the activity of cdc2/cyclin B are apparently involved. For instance, the *p53* gene, which is required for maintenance of the G2 checkpoint via expression of p21, is a major target of genetic alteration in cancer. Likewise, the *ATM* gene (originally identified as the gene mutated in ataxia telangiectasia), which can activate p53 in response to DNA damage, is altered in several other forms of cancer and may be a tumor suppressor [52,75-77]. Chk1 and Chk2, which are downstream of ATM and regulate the activity of the cdc2 phosphatase, cdc25C, have also been found to be genetically altered in human cancer [78-80]. All of these mutations affect the ability of the cell to induce cell cycle checkpoints in response to DNA damage, including the G2 checkpoint, which revolves around regulating the activity of cdc2/cyclin B.

Finally, the importance of both the Rb and p53 pathways in tumorigenesis is emphasized by the mechanisms through which tumor viruses have evolved in order to knock out the functions of these two genes or to alter the activity of other regulators of these pathways. For example, the human papilloma virus (HPV) is involved in development of cervical carcinoma. HPVs express two proteins, E6 and E7, which bind to and knock out the functions of p53 and Rb, respectively [81]. Likewise, the large T antigen of the SV40 virus is a potent viral oncogene that binds to both p53 and Rb to abolish their cellular activities [82]. Oncogenic transformation by human adenoviruses requires the action of two viral genes, *E1A* and *E1B*, which can generate proteins that bind to Rb and p53, respectively, and knock out their cellular functions [83]. In a contrast of mechanism, there is evidence that cytomegalovirus (CMV) induces expression of cyclin E, and that HPV protein E7 can bind to the CKI $p21^{CIP1/WAF1}$ and abrogate its inhibition of both cyclin E- and cyclin A-associated kinase activity [84-86]. Lastly, it has recently come to light that the Kaposi's sarcoma-associated human herpes virus (KSHV/HHV8), believed to be the causative agent of Kaposi's sarcoma and body cavity lymphoma, encodes a cyclin protein most closely related to cyclin D2. This cyclin forms complexes with cdk6 and may deregulate the cell cycle by phosphorylating the CKI $p27^{KIP1}$ and promoting its degradation [87,88].

Biological rationale for inhibition of CDKs as an approach to cancer therapy

It has been stated on numerous occasions in the literature that, since CDKs play a central role in the cell cycle and their activity is often altered in human cancer, then compounds that inhibit CDK activity may have therapeutic value as a cancer treatment [7-12]. More recently, our understanding of the cell cycle at the molecular level has increased to the point that we can now discuss why an inhibitor of one specific CDK versus another (or an inhibitor of more than one CDK) may represent a better anticancer agent or elicit a different response.

Here we present a combined biological and drug development perspective of why inhibition of one or more CDKs is a valid approach to cancer treatment. Selection of a molecular target for cancer therapeutics is usually based on whether the activity of the protein, or the biological pathway where it resides, is found altered or strongly associated with the transformed phenotype or whether it is the target of functional inactivation by transforming viruses. Ideally, this is followed by a series of experiments, which would suggest that correction of the abnormality or inhibition of the pathway would return the cell to the normal state. This 'proof of concept' has been accomplished by such techniques as gene knockout, antisense oligonucleotides, dominant negative (dominant interfering) forms of the protein and antibodies that neutralize the function of the target, to name but a few.

As outlined in the previous section, there is a mass of data to suggest that cyclin D-dependent kinases and their regulators are altered in human cancer as well as evidence for alteration of cyclin D-dependent kinase activity by a potentially transforming virus (Kaposi's Sarcoma virus expresses a viral, cyclin D2-like protein) [89]. Considerable experimental evidence indicates that inhibition of cyclin D-dependent kinase activity will at least partially revert the abnormal/cancer phenotype of a cell. The fact that the CKI, $p16^{INK4A}$, specifically inhibits the activity of cdk4 and cdk6, but not other CDKs [90] has provided researchers with a molecule with which to investigate the effects of cyclin D-dependent kinase inhibition in cells. A number of groups have expressed exogenous $p16^{INK4A}$ in tumor cells, using an adenovirus gene delivery system or an inducible promoter, and found that this blocked their growth and, according to some reports, their tumorigenic potency both *in vitro* and *in vivo* [91-99]. Co-expression of p16 with p53 using an adenovirus gene delivery system was found to induce apoptotic tumor cell death suggesting that cooperation between p53 and inhibition of cyclin D-dependent kinases may make an effective antitumor therapy [100]. However, p16-mediated apoptosis has also been reported in HeLa cells which are functionally deficient for p53 via expression of the E6 papilloma virus protein [101]. Interestingly, ectopic expression of p16 has been shown to increase the sensitivity of human non-small cell lung carcinoma cells to the topoisomerase I inhibitors and radiation, suggesting that a cdk4/cdk6-specific drug may be beneficial when combined with established chemotherapeutic agents [102]. However, in some cells p16 expression appears to protect against cytotoxic agents [103]. A related strategy has been to use a 36-residue peptide, corresponding to amino acids 84 to 103 of p16, coupled to the penetratin (Cyclacel Ltd) peptide carrier molecule. This peptide has the ability to enter cells and inhibit the activity of cdk4 and has been used to show that inhibition of cyclin D-dependent kinases blocks the proliferation of cells with functional Rb [104].

At this point it should be noted that inhibiting the proliferation of a cell by ectopic expression of $p16^{INK4A}$ may not be the same as either blocking cdk4 function using a catalytically inactive form of the kinase (dominant negative) or by using a small molecule inhibitor that binds into the ATP pocket. For instance, expression of $p16^{INK4A}$ in the U2OS osteosarcoma line, which possesses functional Rb, induces a G1 arrest but expression of a dominant negative form of cdk4 does not [105, 106]. This may be because binding of $p16^{INK4A}$ to cdk4 results in a redistribution of members of the CIP/KIP family of CKIs from cdk4 to cdk2 and hence, loss of both cdk4 and cdk2 activity. However, the dominant negative form of cdk4 does not do this. Thus, in some tumors, inhibition of cdk4 activity with a small molecule drug may not be sufficient to permanently arrest a tumor cell in G1. On the other hand, there is a recent publication outlining a screen of the NCI 60 cell-line panel, comparing the growth inhibitory activity of 50,000 compounds with the $p16^{INK4A}$ status of the cells. Those growth inhibitory compounds that correlated highest with $p16^{INK4A}$ deletions or mutations in $p16^{INK4A}$ were found to be fairly selective inhibitors of cdk4, indicating that certain tumors were indeed fully susceptible to inhibitors of this enzyme [107].

In order to assess cdk2 as a target in cancer, it must be considered in the context of whether the enzyme is associated with either cyclin E or cyclin A, since these complexes act at different stages of the cell cycle. With regard to cyclin E, there is evidence that this gene is amplified in a number of tumor types and high cyclin E expression correlates with low $p27^{KIP1}$ levels and poor prognosis in young breast cancer patients [63,108]. There have been numerous experiments showing that ectopic expression of cyclin E induces proliferation in many cell types and, most recently, ectopic expression of cyclin E was shown to cause karyotype instability [68]. Thus, a specific cdk2/cyclin E inhibitor may not only block proliferation but may also have an inhibitory effect on chromosomal instability. Reports of knocking out the activity of cdk2 have mostly been carried out with dominant negative forms of cdk2 or oligonucleotides antisense to cdk2, which would remove both cyclin E- and cyclin A-associated activity. This has been shown to result in a G1 cell cycle arrest and loss of proliferation in transformed cells and, in some cell types, differentiation [105,109]. Expression of cyclin A is high in some tumor types but, as mentioned earlier in this review, the significance of this is unclear. Recent discoveries concerning cyclin A function, however, may provide a rationale for why inhibition of cdk2/cyclin A could be an anticancer treatment.

As G1 progresses and Rb becomes phosphorylated, suppression of E2F/DP1 (E2F) transcriptional activity is eliminated and genes required for S phase are activated. As cells enter S phase, the function of E2F is again suppressed, this time by the cdk2/cyclin A complex that interacts with and phosphorylates E2F, thereby shutting off its DNA binding function [110,111]. Mutational analysis has shown that if E2F can no longer interact with cdk2/cyclin A, or if phosphorylation of E2F by cdk2/cyclin A is blocked, then E2F remains active during the stage in S phase when it is no longer required [112]. This inappropriate activity results in an S phase cell cycle arrest and apoptosis. Therefore, small molecules that block cdk2/cyclin A function during S phase could lead to an S phase checkpoint and cell death. Furthermore, it is known that tumors that have mutations of the Rb pathway have more active E2F complexes and so these cells may be more sensitive to the S phase checkpoint than normal cells. Evidence for this strategy has come to light with the publication of a report describing peptides that could block the interaction between cyclin A and E2F [113,114]. These peptides were found to induce an S phase arrest as well as apoptosis in transformed cells but had a much reduced effect on normal lines, suggesting that this approach may provide a good therapeutic window. Thus, small molecules that can block the interaction of cdk2/cyclin A with E2F or can inhibit the ability of the complex to phosphorylate E2F may provide an approach that selectively kills tumor cells via high levels of active E2F.

Unlike the CDKs or cyclins that we have described thus far, neither cdc2 nor the B-type cyclins appear to be directly altered in human cancer or to be the target of transforming viruses. Most experiments that knock out cdc2 function in the cell have been used to evaluate the effect on hyperproliferation following arterial angioplasty in animal models [115,116]. To date there is evidence that inhibition of cdc2 activity may generate an antiproliferative response but there is no data to suggest that it would preferentially kill tumor cells. However, it should be mentioned that some of the molecules that regulate the G2 DNA damage checkpoint and hence, the cdc2/cyclin B kinase activity, such as p53, ATM and Chk2, are altered in cancer (see previous section). These alterations could potentially lead to inappropriate activation of cdc2 in this scenario.

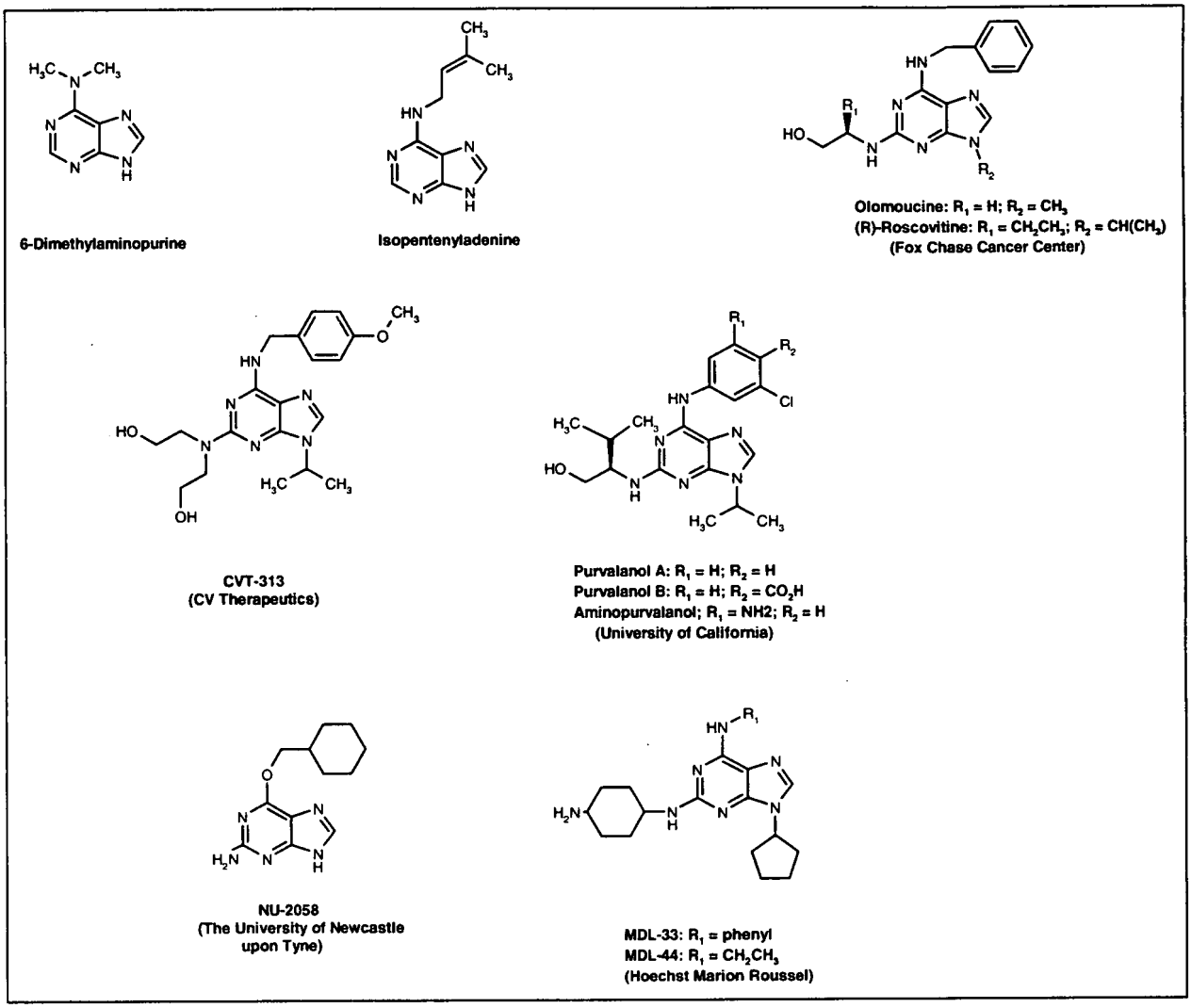
Finally, there is the issue as to whether inhibition of a single, specific CDK or inhibition of more than one CDK would have the greater beneficial effect as an anticancer treatment. Looking at G1 progression and cancer, it is not yet clear whether inhibition of the catalytic activity of just cdk4 with a drug would be sufficient to invoke a G1 arrest, or whether inhibition of cdk2/cyclin E would also be required [106]. In counterpoint to that argument, cdk4 inhibitors were indeed identified in a screen of tumor cell lines where inhibition of growth correlated with $p16^{INK4A}$ status, suggesting that such a drug would block proliferation [107]. Whether the block is a G1 arrest has yet to be reported. Mechanistically, a broad-spectrum CDK inhibitor would block cells in multiple phases of the cell cycle, which could present two counteracting therapeutic possibilities. Such an inhibitor might cause additional stress to tumor cells and offer further cell killing potential. In contrast, arresting cells at multiple sites may prevent tumors from entering the very phase of the cell cycle in which they are most prone to dying and thus, a protective effect might result. Experimental data obtained with current broad-spectrum inhibitors has supported the first possibility and evidence indicates that these compounds are cytotoxic (see next section). One obvious liability to accepting a less specific inhibitor is a potential for increased toxicity.

Chemical inhibitors of CDKs

Purine analogs

The earliest inhibitors of cyclin-dependent kinases (CDKs) were purine-based structures (Figure 2), which had modest inhibitory activity against cdc2/cyclin B. These initial compounds included 6-dimethylaminopurine (IC_{50} = 120 µM)

Figure 2. Purine analogs.



and subsequently isopentenyladenine (IC_{50} = 55 μM) and olomoucine (IC_{50} = 7 μM) [117]. The latter compound exhibited moderate selectivity and was much less potent against cdk4/cyclin D1 and cdk6/cyclin D3 as well as against other kinases, including protein kinase C (PKC) and insulin receptor tyrosine kinase [117]. Further screening of additional analogs identified roscovitine (Fox Chase Cancer Center), in which the 2- and 9-substituents were modified, producing a 10-fold increase in potency against cdc2 and cdk2 [118]. These structural modifications also improved cellular activity with an average IC_{50} = 16 μM against the NCI tumor panel [15]. Cells treated with both olomoucine and roscovitine arrested in G1 and G2/M phases of the cell cycle [119-122] and exhibited other biological effects that were consistent with inhibition of CDKs. However, the concentrations necessary to produce these cellular effects were extremely high and more than likely out of the range to maintain specificity.

Co-crystallographic studies of isopentenyladenine, olomoucine and roscovitine show that these compounds do indeed bind into the ATP pocket of cdk2 [123•,124-125]; however, the orientation was unexpectedly different from the purine ring of ATP. By capitalizing on the structural information obtained from the cdk2-inhibitor co-crystals and applying computer-aided drug design, a modest but directed combinatorial library of purine analogs was generated and screened against cdk2/cyclin A [126,127] as well as a number of other purine analogs synthesized by conventional methods [201,202]. These efforts produced a series of purine analogs with submicromolar activity against the enzyme, one of which was CVT-313 (CV Therapeutics Inc; Figure 2). The biological properties of this particular analog have been characterized. It was a competitive inhibitor of ATP with an IC_{50} value of 0.5 μM and a K_i value of 0.095 μM against cdk2/cyclin A. The compound exhibited modest selectivity versus other CDKs, with 8.5- and 430-fold lower activity against cdc2 and cdk4, respectively, and little or no activity against MAP kinase or PKC. CVT-313 caused biological effects consistent with inhibition of CDK activity in that it caused a reduction in Rb phosphorylation, was growth inhibitory and arrested proliferating cells at the G1/S boundary and in G2/M [128]. The antiproliferative effects against tumors occurred at concentrations ranging from 4 to 20 μM. CVT-313 was tested in a carotid artery model of restenosis where a brief intraluminal exposure to a denuded rat carotid artery resulted in > 80% inhibition of neointima formation.

Further syntheses of purine libraries, in which the 2, 6, and 9 substituents were separately varied, resulted in several compounds with low nanomolar potency against cdk2 [203,204]. The most potent was purvalanol B (Figure 2), which had an IC_{50} value of 6 nM against cdk2/cyclin A [129••], corresponding to a > 1000-fold increase in potency over olomoucine. A more membrane-permeable analog, purvalanol A (Figure 2), had an IC_{50} value of 70 nM against cdk2/cyclin A and both A and B had exceptional activity against cdc2/cyclin B, with IC_{50} values of 4 and 6 nM, respectively. Purvalanol exhibited excellent specificity toward the CDKs and had little or no inhibitory activity against a large panel of serine/threonine and tyrosine kinases. The crystal structure of the human cdk2/purvalanol B complex was solved to a resolution of 2.05 Å [129••]. The molecule fits efficiently into the ATP-binding site and the overall geometry resembled that of inhibitor-complexes involving the related inhibitors, olomoucine and roscovitine, with the C^2, N^6 and N^9 substituents occupying similar binding pockets. Purvalanol A was tested against the NCI tumor panel of 60 cell lines and the average GI_{50} (concentration of compound necessary to inhibit growth by 50%) was 2 μM. The *in vitro* biological properties of purvalanol A have been characterized in the U937 human histocytic lymphoma cell line [130]. The IC_{50} for growth inhibition was 7.5 μM and analysis by flow cytometry showed that 5 μM purvalanol A caused cells to arrest in the G2/M phase of the cell cycle. The lack of purvalanol-treated cells in mitosis, as assessed by microscopic inspection, was consistent with the cells being blocked in G2. Concentrations of purvalanol A higher than 10 μM caused the cells to enter apoptosis.

Along these same lines, a very close analog, aminopurvalanol (Figure 2), has been further characterized with respect to its biological properties. This compound inhibited cdk2/cyclin A and cdc2/cyclin B with equal potency from the extracts of U937 cells; however, the biological effects in intact cells were consistent with cdc2/cyclin B as the dominant target for this compound [131]. Cells exposed to 5 μM aminopurvalanol caused an increase in G2/M as early as 8 h after the beginning of treatment. In dose-response and time-course experiments, aminopurvalanol treatment caused a progressive increase in the G2/M phase with a concomitant decrease in the G1 phase and a relatively constant S phase, indicating that cells were free to cross the G1 to S boundary. Microscopic examination indicated that < 1% of the treated cell population was undergoing mitosis, indicating that the block was in G2. Prolonged exposure to aminopurvalanol over 4 days caused the U937 cells to exhibit characteristics of differentiated macrophages and neutrophils, as assessed by reduction of nitroblue tetrazolium (NBT) and expression of CD11b integrin. Additional evidence to indicate that cdc2/cyclin B was the major target of aminopurvalanol was obtained with a close structural analog of aminopurvalanol, bearing a 4-carboxyl group attached via a short linker to an agarose-based solid support. This system was used to isolate proteins that bound to the inhibitor from cellular extracts and indicated that cdc2 was the major CDK bound to the matrix, whereas very little cdk2 was present.

A series of 6,9-di-substituted-2-[trans-(4-aminocyclohexyl) amino]purines was recently reported by Hoechst Marion Roussel to have excellent potency as inhibitors of cdk2/cyclin E activity [205,206]. Potencies as low as 0.01 μM against cdk2/cyclin E were reported and selectivity with respect to cdk4/cyclin D1 was > 100-fold. One of the more potent compounds against these enzymes was MDL-44 (Figure 2) [206], which had IC_{50} values of 0.01 μM against cdk2/E and 0.25 μM against cdk4/D1. This compound had antiproliferative activity, with IC_{50} values ranging from 0.5 to 0.85 μM against the MDA-MB 231, MCF-7 and MDA-MB-435 human breast carcinomas, the HT-29, HCT-15, and colo205 human colon carcinomas, the A549 and DMS-114 human lung carcinomas and the PC-3 and DU145 human prostate carcinomas. MDL-33 (Figure 2) [205] had excellent

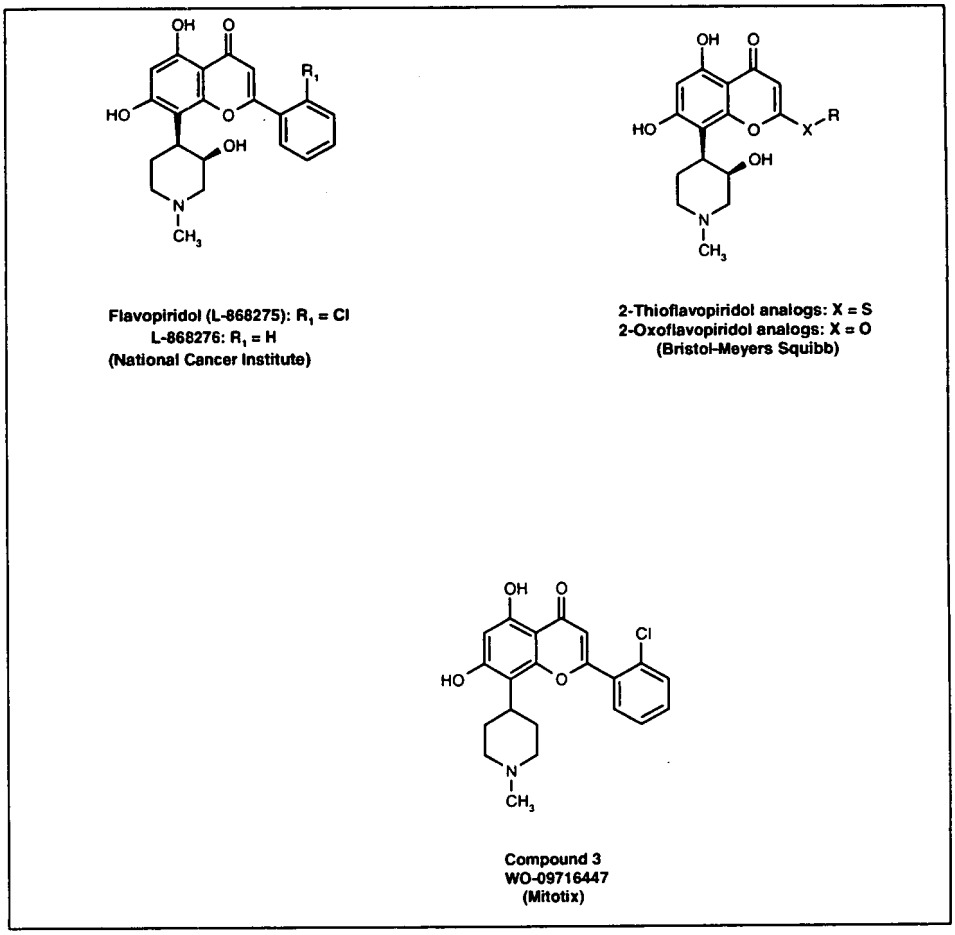
antiproliferative activity, with IC_{50} values against tumor growth of between 0.12 and 0.27 µM. PC3 human prostate tumor cells were blocked in the G1 and S phase of the cell cycle, and phosphorylation of Rb in the MCF-7 human breast carcinoma was prevented after these cells were exposed to MDL-33 for 24 h [132].

Finally, another series of purine analogs, which were modified mainly at the 6 position, have also been recently disclosed as CDK inhibitors [207]. NU-2058 (The University of Newcastle upon Tyne; Figure 2) was reported to have an IC_{50} of 6 µM against cdc2/cyclin B and 16 µM against cdk2/cyclin A3 [133,134]. A co-crystal structure of NU-2058 with cdk2 showed hydrogen bonding between the N^9, N^3 and 2-NH_2 positions with Glu81/Leu83 [135].

Flavones

The most advanced CDK inhibitor in terms of clinical development is flavopiridol (L-868275, NSC-649890; National Cancer Institute; Figure 3). This compound is a synthetic flavone structurally related to a natural alkaloid and was originally purified from the stem bark of *Dysoxlum binectariferum*, a plant indigenous to India [136]. It has been reported to inhibit cdc2, cdk2, cdk4 and cdk7, with IC_{50} values in the range of 0.4 µM, and immunoprecipitated CDK activities from cells, with IC_{50} values in the range of 0.1 to 0.4 µM [136-138]. Flavopiridol not only inhibited the catalytic activity of these CDKs, but also inhibited activating phosphorylation events on the enzymes themselves [139,140]. There is also some evidence that flavopiridol downregulates cyclin D1, which would also contribute to a decline in cdk4 activity [141]. L-868276, the deschloro derivative of flavopiridol (National Cancer Institute; Figure 3), has been cocrystallized with cdk2, laying out the binding orientation of the aromatic group in the ATP binding pocket. The position of the phenyl group allows contact points with the enzyme not observed with ATP and provides a rationale for its specificity toward CDKs [142]. Flavopiridol has been shown to be cytostatic in some tumor cells, causing arrest in both G1 and G2 phases of the cell cycle [137,143-145]. The compound, however, was also cytotoxic in many epithelial [144-148] and hematopoietic [149-151] tumor models, causing a well-defined apoptotic response. Several studies have shown flavopiridol to have significant *in vivo* antitumor activity, leading to partial regression of a variety of human tumors grown as sc and subrenal xenografts in nude mice [144,150-155]. Many of the biological effects of flavopiridol can be attributed to inhibition of CDKs; however, because this agent caused cell death equally well in both cycling and non-cycling cell populations [147], some questions have been raised as to whether it is really specific toward CDKs and whether its cytotoxic activity might involve additional mechanisms [156-158].

Figure 3. Flavone analogs.



Flavopiridol is presently in clinical trials [156,159•]. Initial studies defined a maximal tolerated dose of 50 mg/m^2/day, with diarrhea as the dose-limiting toxicity (DLT) [159•]. There was, however, no evidence of mucosal damage in the form of ulceration, bleeding or inflammation. When dose escalation was allowed in the presence of antidiarrheal prophylactic medication, a reversible hypotension became the DLT. An unexpected finding showed that flavopiridol caused a dose-related inflammatory syndrome, giving rise to flu-like symptoms. Plasma concentrations of 200 to 400 nM were achieved during this clinical trial, which were well within the range needed to inhibit CDKs and tumor growth. One partial (> 50% tumor shrinkage) and three minor (< 50% shrinkage, but sustained for > 3 months) responses were reported. Similar data were obtained from another clinical trial employing 72-h infusions [160]. Phase II trials are also in progress, with the intention of combining flavopiridol with conventional cytotoxic agents. *In vitro* studies indicate that maximum therapeutic enhancement occurs when the cytotoxic agent is given first, followed by flavopiridol [156]. Based on preclinical evidence that flavopiridol might augment paclitaxel-induced apoptosis [161], a combination phase II trial was initiated where paclitaxel was first infused for 24 h followed by a 24-h infusion of flavopiridol [162]. Evidence of an antitumor effect was obtained in patients with esophageal and prostate carcinoma. A phase II study in patients with renal carcinoma was completed on the 72-h infusion schedule, in which a single partial response was noted [163]. Recent clinical data, however, indicate more disappointing results [164].

Bristol-Myers Squibb has synthesized a series of 2-thio and 2-oxo analogs of flavopiridol as inhibitors of CDKs (Figure 3) [208]. Some of the compounds had potent inhibitory activity against cdc2/cyclin B, with IC_{50} values as low as 150 nM, and excellent selectivity (50- and 150-fold lower potency against cdk2 and cdk4, respectively). Additional data implicating cdc2/cyclin B as the specific target were obtained from cell lines that were selected for resistance to these compounds. The cells were ~150-fold resistant to thioflavopiridol in soft-agar assays and were found to overexpress cdc2 to a high degree. In general, these compounds were less cytotoxic than flavopiridol, but were equally potent in preventing tumor clone formation in soft agar, with IC_{50} values in the range of 1 to 50 nM. In addition, cells lacking p53 function were up to 25-fold more sensitive to growth inhibition by these compounds [165].

Finally, Mitotix has synthesized a large number of flavopiridol analogs in the hope of improving therapeutic properties within this chemical class [209,210]. One example that was highlighted in their patents was compound 3 (Figure 3). The inhibitory activity against cdk4/cyclin D was given as < 50 μM and an actual IC_{50} value was not provided. The compound was inhibitory at 0.2 μg/ml against three breast carcinomas, BT 549, MDA-MB-453 and MCF-7.

Pyrimidines

CGP-60474 (Novartis AG – discontinued; Figure 4) is a phenylaminopyrimidine derivative that inhibited cdc2 and cdk2 with IC_{50} values of 0.020 and 0.050 μM, respectively, and was inactive against cdk4 [166]. This compound exhibited good selectivity in that it was less potent against PKC isozymes, c-fgr, and erk-1 and inactive against JNK, p38 and p70-S6, as well as EGF, PDGF, IGF-1 and insulin-mediated protein phosphorylation in cells. CGP-60474 displayed antiproliferative activity against a variety of tumor cell lines, with IC_{50} values ranging from 0.01 to 0.1 μM. The compound bound in the ATP pocket of cdk2 but also had the unusual property of dissociating all cdk2/cyclin A and E and cdc2/cyclin B complexes [167]. In addition, Rb(-) and p53(-) cells were induced to undergo apoptosis by the presence of CGP-60474 [167].

Another series of pyrimidines with more modest activity against cdc2 and cdk2 was also reported [211]. NU-6027 (The University of Newcastle upon Tyne; Figure 4) produced IC_{50} values of 2.6 μM and 2.2 μM, respectively.

Pyridopyrimidines

Warner-Lambert disclosed a series of pyrido[2,3-d]pyridopyrimidines and 4-aminopyrimidines, which have remarkable potency and specificity toward one or more CDKs [212••]. IC_{50} values as low as 0.004, 0.01 and 0.06 μM were reported against cdk4/cyclin D1, cdk2/cyclin A and cdc2/cyclin B, respectively. Many of the compounds exhibited excellent specificity for CDKs versus other protein kinases. Likewise, within the CDKs, good specificity between cdk4, cdk2 or cdc2 was obtained, with selectivity ratios ranging from 10- to 40-fold. Two compounds from this series have been highlighted in public presentations. PD-171851 [168••] (Figure 5) inhibited purified cdk4 with an IC_{50} of 0.042 μM while exhibiting 20- and 30- fold lower activity against cdk2 and CDC2, respectively, and little or no activity against other serine/threonine or tyrosine kinases. This

Figure 4. Pyrimidine analogs.

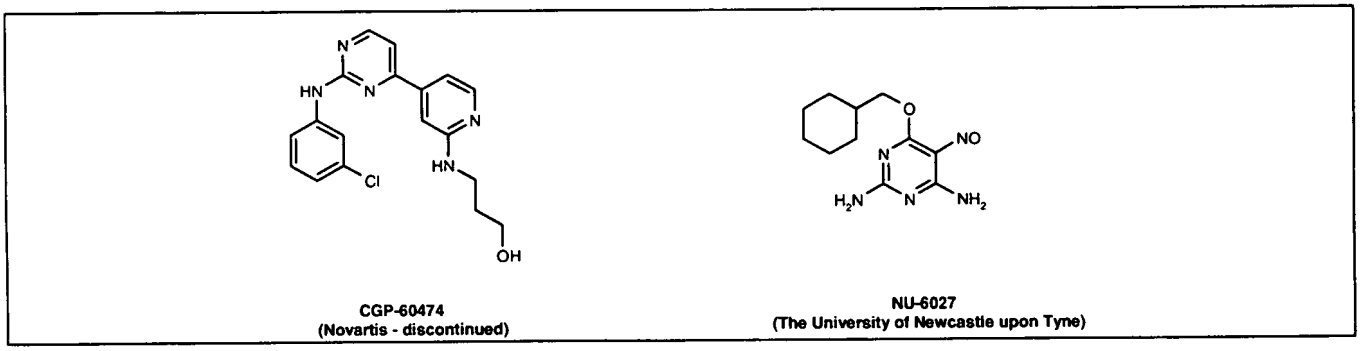
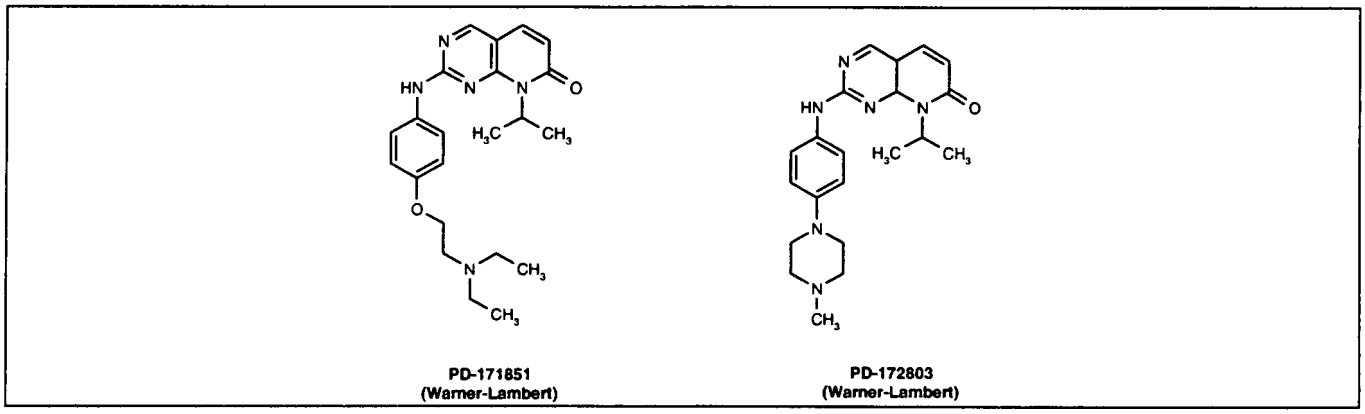


Figure 5. Pyrimidopyrimidines.

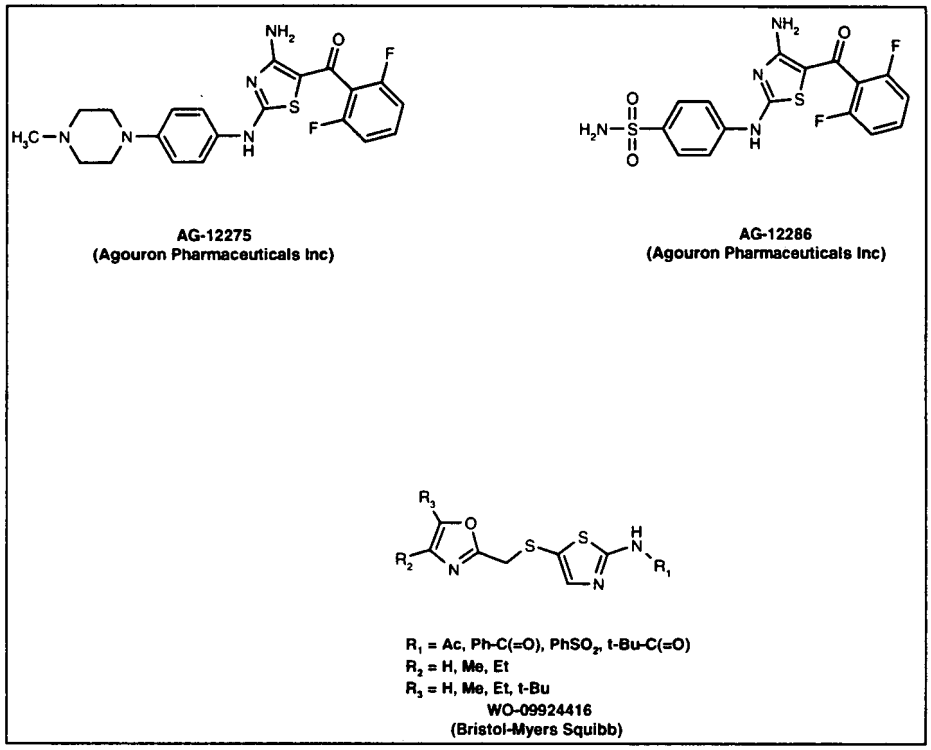


series of compounds exhibited pure competitive inhibition with respect to ATP and non-competitive inhibition with respect to Rb. Flow cytometry analysis of several tumor models combined with bromo-deoxyuridine incorporation showed that Rb+ cells treated with PD-171851 specifically arrest in G1 whereas Rb– cells continue to cycle. The cell cycle effects of PD-171851 occurred at the same concentration range as that for growth inhibition (IC_{50} = 0.8 μM for MCF-7 breast carcinoma). Prolonged treatment of tumor cells with PD-0171851 did not cause cell death but induced morphological changes in terms of a flattened, more differentiated phenotype. Another analog, PD-172803 (Figure 5), inhibited cdk4/cyclin D1 with an IC_{50} value of 0.032 μM [212••] and selectively reduced the phosphorylation level on Rb at specific cdk4 sites [169••].

Thiazoles

Agouron has reported a large number of 4-aminothiazole derivatives as inhibitors of CDKs [213••]. Many of these compounds are extremely potent inhibitors of cdk4/cyclin D3 and/or cdk2/cyclin A and certain compounds have excellent specificity toward cdk4 [170-173••]. AG-12275 (Figure 6) had a K_i of 0.003 μM against cdk4/cyclin D3 and 0.22 μM and 0.32 μM against cdk2/cyclin A and cdc2/cyclin B representing selectivity ratios for cdk4 of 70- and 100-fold, respectively. Other compounds were more broad-spectrum inhibitors, such as AG-12286 (Figure 6), which had K_i values of 13, 6, and 2 nM against cdk4, cdk2 and cdc2, respectively. Both AG-12275 and AG-12286 bound in the ATP pocket of the enzyme and exhibited good specificity, with little activity against non-CDK protein kinases [170-173••]. These

Figure 6. Thiazoles.



compounds inhibited the proliferation of a variety of tumor cell lines, including the HCT116, COLO-205 and SW-480 colon carcinomas, the U2-OS and Saos-2 osteosarcomas, and the MCF-7 and MDA-MB-468 breast carcinomas at submicromolar concentrations. AG-12286 caused a cell cycle block in both the G1 and G2 phases whereas AG-12275 caused G1 arrest that was dependent on functional Rb. Both compounds reduced phosphorylation of Rb at Ser780 *in vitro* and delayed the growth of HCT-116 human colon carcinoma xenografts in nude mice [170-173••].

Bristol-Myers Squibb have also reported a large series of aminothiazoles (Figure 6) that have been claimed as inhibitors of CDKs. No data were given except that IC_{50} values for many of the compounds were < 50 μM [214].

Oxindoles

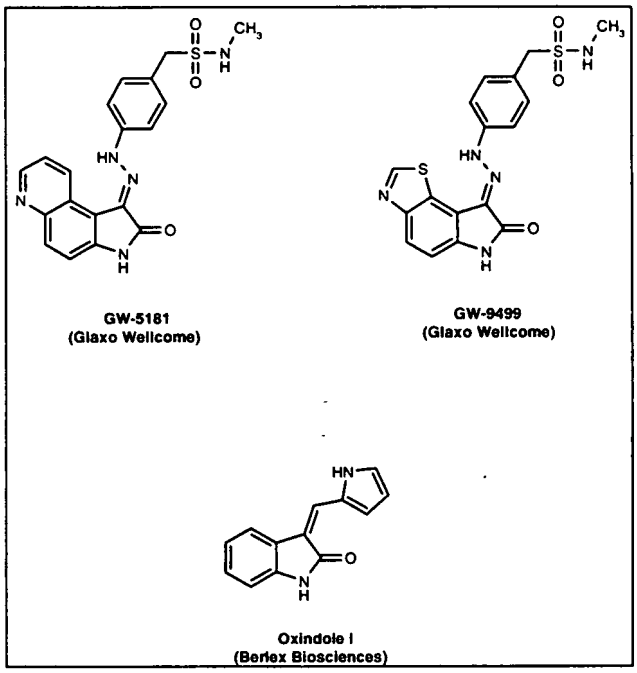
A series of substituted oxindole derivatives were disclosed by Glaxo Wellcome as inhibitors of cdk2 and cdc2, with the best inhibitors having IC_{50} values of 1 to 10 nM for inhibiting purified enzyme preparations and < 1 μM for inhibiting cell cycle progression [215•]. GW-5181 and GW-9499 (Figure 7) inhibited cdk2/cyclin A activity with IC_{50} values of 6 and 3 nM, respectively, and have been co-crystallized with cdk2, thus providing information on the binding mode for this enzyme-inhibitor complex [174,175]. Both compounds induced a cell cycle block in cells, inhibiting DNA synthesis and resulting in the accumulation of cells in the G1 and G2 phases of the cell cycle. GW-9499 inhibited growth of human diploid fibroblasts (HDF), colon carcinoma cells (RKO) and MDA-MB-468 human breast carcinoma, with IC_{50} values of 8, 0.86 and 3.4 μM, respectively. Prolonged exposure (4 to 6 days) at 5 μM resulted in cell death for the RKO and MDA-MB-468 cells. GW-5181 produced a moderate decrease in the growth rate of RKO colon tumor cells grown as xenografts in nude mice, when administered at 100 mg/kg/day iv for 10 days.

Berlex Biosciences reported on four compounds with very modest activity against cdk4/cyclin D1, including an oxindole, two ureas and a benzoic acid derivative [176]. Despite the modest activity against cdk4 (IC_{50} = 4.9 μM], oxindole 1 (Figure 7) exhibited submicromolar inhibitory activity against the Rb(+) cell lines, MCF-7 and ZR-75-1, with IC_{50} values of 0.42 and 0.10 μM, respectively. This compound was less active against the Rb-negative cell line, BT-549, at 13.9 μM, which is consistent with its mechanism being inhibition of cdk4.

Butyrolactone-1

Butyrolactone-1 (Figure 8) is a natural product isolated from the culture medium of *Aspergillus* species F25799. It was identified as an inhibitor of murine cdc2/cyclin B, with an IC_{50} of 0.68 μM [177]. The compound was equally active against cdk2 and exhibited reasonable specificity, with little activity against MAP kinase, protein kinase C, protein kinase A, casein kinase or the EGF receptor tyrosine kinase. Butyrolactone-I inhibited Rb and histone phosphorylation and prohibited cell cycle progression in WI-38 human lung fibroblasts by preventing cells in G1 from traversing into S phase as well as progression through G2/M [178]. Fairly high concentrations were necessary to elicit these cellular effects and experiments in permeabilized cells indicate that

Figure 7. Oxindoles.



the plasma membrane is poorly permeable to this compound [179]. Nevertheless, inhibition of cell cycle progression by butyrolactone-I has been demonstrated in several tumor cell lines derived from different tissue types [179-181].

Paullones

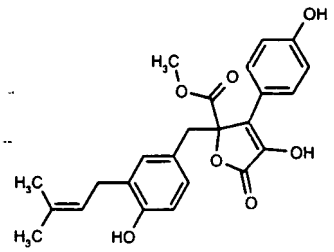
The paullones were discovered at the National Cancer Institute using the COMPARE algorithm, which is a computerized pattern recognition tool for identifying novel compounds that have a mechanism of action or biochemical target similar to a reference compound [182]. Applying this strategy and using flavopiridol as a reference, 9-bromo-7,12-dihydroindolo[3,2-*d*][1]benzazepin-6(5H)-one (kenpaullone; Figure 8) was identified as an inhibitor of CDKs. Kenpaullone is an ATP-competitive inhibitor and inhibited cdc2/cyclin B with an IC_{50} value of 0.4 μM. It displayed almost equal activity against cdk2/cyclin A (IC_{50} = 0.68 μM) and cdk5/p35 (IC_{50} = 0.85 μM) [183•] but had little activity against cdk4/cyclin D1. The compound had excellent selectivity toward CDKs with little or no activity against a panel of 20 different tyrosine and serine/threonine protein kinases. Using coordinates from crystal structures previously published for cdk2 with bound ATP or inhibitors, a molecular model was developed which indicated a binding mode similar to that of olomoucine and roscovitine. Major contacts include hydrogen bonds to both the backbone carbonyl and amide of Leu83 and positioning of ring atoms between the Leu134 and Ile10 hydrophobic side-chains, with the D ring of the inhibitor occupying a hydrophobic pocket formed mainly by Phe80, Val18, Ala144 and Lys33 [183•]. Kenpaullone had only modest antiproliferative activity against tumor cells, with an average IC_{50} for growth inhibition in the NCI tumor panel of 43 μM. However, replacement of the 9-bromo substituent with hydrophilic, electron-withdrawing substitutions resulted in dramatically improved enzyme potency. Alsterpaullone (Figure 8) exhibited an IC_{50} of 0.035 μM against cdc2/cyclin B and inhibited the proliferation of HCT-116 human colon carcinoma with an average IC_{50} of 0.12 μM [184]. This dramatic increase in potency plus the fact that

kenpaullone occupies very little of the pocket in CDKs where the ribose and phosphate groups of ATP resides (and therefore could be expanded into), implies this chemical class of inhibitors has considerable potential for improvement through structural modification.

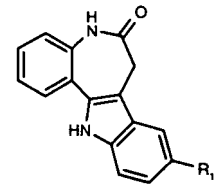
Indirubins

Recent data indicate that analogs of a natural product, indirubin (Figure 8), which exhibits antileukemic activity, are inhibitors of CDKs [185]. This raises the possibility that inhibition of CDKs may contribute to the antitumor effects of these compounds. Indirubin-5-sulphonic acid was the most potent inhibitor of CDKs, with IC_{50} values of 0.055, 0.035, 0.15, 0.3 and 0.065 μM, respectively, against cdc2/cyclin B, cdk2/cyclin A, cdk2/cyclin E, cdk4/cyclin D1 and cdk5/p35. The 3'-monoxime was 2- to 3-fold less potent. These compounds appear to have considerable specificity against CDKs, with little activity against a large panel of other protein kinases [185]. Both indirubin-3'-monoxime and indirubin-5-sulphonic acid have been co-

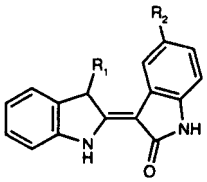
Figure 8. Chemical structures of additional cyclin-dependent kinase inhibitors.



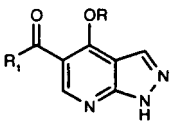
Butyrolactone-I



Kenpaullone: R_1 = Br
Alsterpaullone: R_1 = NO_2
(National Cancer Institute)

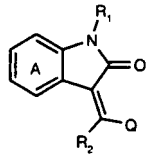


Indirubin: R_1 = O; R_2 = H
Indirubin-3'-monoxime: R_1 = NOH; R_2 = H
5-Chloro-indirubin: R_1 = O; R_2 = Cl
Indirubin-5-sulphonic acid: R_1 = O; R_2 = SO_3H

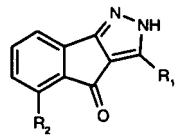


R = BuO, cyclohexylmethoxy, $PhCH_2O$, BuS, Bu-S(=O)-, $BuSO_2$
R_1 = 2,4,6-F_3-Ph, 2,3,5,6-F_4-4-Me-Ph, NH_2, Ph

WO-09930710
(Bristol-Myers Squibb)

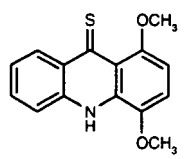


Ring A is opt subst and may contain an N atom
Q = opt subst aryl, heteroaryl, heterocyclylyl, carbocyclyl
R_1 = H, alkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, -C(=O)-CF_3, heterocyclyl, OH, alkoxy, acyl, alkoxycarbonyl -C(=O)-NH_2, -C(=S)-NH_2, NH-C(=O)-NH_2, -C(=NH)-NH_2, NH-C(=NH)-NH_2, alkylsulfonyl, arylsulfonyl, trihalomethanesulfonyl
R_2 = H, alkyl, cycloalkyl, aryl, heteroaryl, heterocyclyl, halo
WO-09850356
(Sugen)

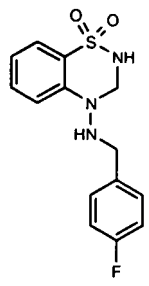


R1 = alkyl, cycloalkyl, opt subst Ph, heteroaryl
R2 = H-C(=O)-NH, R_3-CH_2-C(=O)-NH-, Ph-C(=O)-NH, heteroaryl-C(=O)-NH-, alkyl-NH-C(=O)-, cycloalkyl-NH-C(=O)-, Ph-NH-C(=O)-, heterocyclyl-NH-C(=O)-, H_2N-C(=O)-NH-, H_2N-NH-C(=O)-NH-

WO-09954308
(Du Pont)



NSC-625987
(USA)



NSC-645787
(USA)

crystallized with cdk2 and show that the indirubin derivatives form an extra hydrogen bond with the cdk2, as compared to other inhibitor/cdk2 structures that have been resolved. Similarly to isopentenyladenine, staurosporine (Kitasato Institute) and ATP, these compounds form hydrogen bonds with the peptide oxygen of Glu81 through the lactam amide nitrogen and with the amide group of Leu83, with the lactam amide oxygen. However, the binding mode of indirubins also has some characteristics reminiscent of olomoucine and roscovitine in that they interact with the backbone oxygen of Leu83 through the cyclic nitrogen [185].

Although it was the most potent derivative for inhibiting CDKs, indirubin-5-sulphonic acid had little effect on cells, presumably because of poor permeability. The 3'-monoxime was growth inhibitory against a number of cell lines *in vitro*, starting at concentrations in the 5 to 10 μM range. Flow cytometry analysis shows that the most consistent effect was a G2/M block at concentrations of ≥ 10 μM. However, in some cell lines, notably the Jurkat T-lymphoblast and the MCF-7 breast carcinoma cell lines, an increase in the G1 phase of the cell cycle occurred at lower concentrations. A decrease in the phosphorylation level at Ser807 and Ser811 of Rb was concomitant with these cell cycle effects. Since both indirubin and its derivatives bind to DNA, it is still questionable as to whether inhibition of CDKs plays a significant role in the antitumor activity observed for this chemical class of compounds.

Additional inhibitors of Cdks

A number of companies have disclosed a variety of structural classes of compounds purported to inhibit CDKs. The generic structures are shown in Figure 8. Bristol-Myers Squibb disclosed a series of pyrazolo[3,4-b]pyridines that are reported to inhibit cdk4/cyclin D1, cdk2/cyclin E and cdc2/cyclin B1, with IC_{50} values of < 50 μM [216]. Sugen has reported on 2-indolinone derivatives that have activity against cdk2, with certain compounds having IC_{50} values of < 0.78 μM [217]. Du Pont has disclosed a series of 5-aminoindeno[1,2-c]pyrazol-4-ones as CDK inhibitors [218]. Finally, the NCI has identified two series of molecules through screening over 50,000 compounds for growth inhibitory effects against their panel of 60 cell lines and correlating with the deletion or inactivation status of $p16^{INK4A}$ [107•,219]. This exercise identified 3-aminothioacridone (NSC-680434) and a benzothiadiazine (NSC-645787) as inhibitors of cdk4/cyclin D. Subsequent substructure screening of analogs related to these initial compounds against cdk4 activity identified a more potent acridone, NSC-625987, and benzothiadiazine, NSC-645787 (both in Figure 8). These compounds inhibited cdk4/cyclin D1 activity with IC_{50} values of 0.20 and 0.33 μM, respectively, with no inhibitory activity against cdc2 or cdk2 at concentrations as high as 100 μM. The compounds were growth inhibitory against a number of tumor types in the tumor panel of 60 cell lines; however, no evidence was provided that the growth inhibition was specifically due to inhibition of cdk4.

Conclusion

The concept that suppressing the activity of certain CDKs represents a valid approach to cancer chemotherapy has been flourishing for over five years now. The rationale of pursuing CDKs as targets for anticancer agents has been endorsed by many of the scientists working in this area, as well as by pharmaceutical companies. The justification continues to be strengthened as new evidence emerges linking abnormalities in the cell cycle machinery with the tumorigenic process. The development of inhibitors of CDKs has been an evolutionary process and many of the inhibitors initially discovered suffered from a lack of potency and specificity, which perhaps compromised attempts to demonstrate antitumor activity *in vivo* based solely on their target. However, the field is being revolutionized through the application of new discovery tools, including high-throughput screening methods, combinatorial chemistry, more and higher quality X-ray crystal structures and molecular modeling. These efforts have resulted in a plethora of novel structures, which demonstrate an increase in potency over earlier inhibitors of several orders of magnitude. They exhibit improved selectivity to the point where resulting biological effects can be assigned to CDK inhibition with much more confidence. Indeed, many of these inhibitors are showing signs of *in vivo* antitumor activity, which can be related to target modulation.

As the selectivity toward specific CDKs improves, there will be opportunities to answer questions regarding the utility of individual CDKs as cancer targets versus more broad-spectrum inhibition of several CDKs. One of the more provocative questions is whether selective inhibition of cdk4 will be therapeutic and whether inhibitors of this enzyme will be valuable in combination with other clinically useful cytotoxic agents. Existing data imply that selective inhibition of cdk4 would be purely cytostatic, and studies using engineered tumor models with elevated p16 levels indicate that inhibition of cdk4 in this manner blocks cells in G1, which may protect certain cells from cytotoxic agents but results in apoptosis in other cell types. Then again, a pure cytostatic agent with low toxicity might have excellent utility in cancer, perhaps by keeping a tumor in check while the patient recovers from cytotoxic chemotherapy or radiation therapy. The true utility of a pure cdk4 inhibitor will become apparent in clinical trials. Since many human tumors appear to utilize abnormalities connected with cdk4 function and regulation as part of their neoplastic phenotype, inhibiting this enzyme in the natural background of human patients may eliminate enough of its tumorigenic properties to prohibit abnormal growth and tumor development. Alternately, the recent evidence suggesting that specific inhibition of cdk2 would produce an apoptotic response in tumors (see reference 113) is also quite intriguing and certainly makes this approach worth investigating. Many of the more broad-spectrum inhibitors, including flavopiridol, tend to be cytotoxic and cause cells to enter apoptosis in a manner similar to many other clinically used anticancer agents. These CDK inhibitors have the potential to be utilized alone or in combination, similarly to other cytotoxic agents used clinically. Finally, there is some evidence for differential effects on non-transformed cells versus tumor cells, whereby the former are blocked in one phase of the cell cycle but tumor cells are not. This could be a way of protecting host tissue from the cytotoxic effects of cancer agents while leaving the tumor susceptible. Although the true utility of CDK inhibitors in the clinic will have to be determined empirically, the current concept of the cell cycle and the biological effects demonstrated thus far with

existing inhibitors do provide some basis to be optimistic. As these newer generation-compounds move toward clinical trials, it is evident that the next several years will determine the value of CDK inhibitors as cancer agents.

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Special Lecture

The Pezcoller Lecture: Cancer Cell Cycles Revisited

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Abstract

Genetic lesions that disable key regulators of G_1 phase progression in mammalian cells are present in most human cancers. Mitogen-dependent, cyclin D-dependent kinases (cdk4 and cdk6) phosphorylate the retinoblastoma (Rb) tumor suppressor protein, helping to cancel its growth-inhibitory effects and enabling E2F transcription factors to activate genes required for entry into the DNA synthetic phase (S) of the cell division cycle. Among the E2F-responsive genes are cyclins E and A, which combine with and activate cdk2 to facilitate S phase entry and progression. Accumulation of cyclin D-dependent kinases during G_1 phase sequesters cdk2 inhibitors of the Cip/Kip family, complementing the effects of the E2F transcriptional program by facilitating cyclin E-cdk2 activation at the G_1-S transition. Disruption of "the Rb pathway" results from direct mutational inactivation of Rb function, by overexpression of cyclin D-dependent kinases, or through loss of $p16^{INK4a}$, an inhibitor of the cyclin D-dependent kinases. Reduction in levels of $p27^{Kip1}$ and increased expression of cyclin E also occur and carry a poor prognostic significance in many common forms of cancer. The ARF tumor suppressor, encoded by an alternative reading frame of the *INK4a-ARF* locus, senses "mitogenic current" flowing through the Rb pathway and is induced by abnormal growth promoting signals. By antagonizing Mdm2, a negative regulator of the p53 tumor suppressor, ARF triggers a p53-dependent transcriptional response that diverts incipient cancer cells to undergo growth arrest or apoptosis. Although ARF is not directly activated by signals that damage DNA, its loss not only dampens the p53 response to abnormal mitogenic signals but also renders tumor cells resistant to treatment by cytotoxic drugs and irradiation. Lesions in the p16 — cyclin D-CDK4 — Rb and ARF — Mdm2 — p53 pathways occur so frequently in cancer, regardless of patient age or tumor type, that they appear to be part of the life history of most, if not all, cancer cells.

Introduction

"The greatest single achievement of nature to date was surely the invention of the molecule of DNA. We have had it from the very beginning, built into the first cell to emerge, membranes and all, somewhere in the soupy water of the cooling planet three thousand million years or so ago. All of today's DNA, strung through all of the cells of the earth, is simply an extension and elaboration of that first molecule. In a fundamental sense, we cannot claim to have made progress, since the method used for growth and replication is essentially unchanged" (1).

As the late Lewis Thomas implied, the principle task of the cell division cycle is to replicate DNA (without errors during S phase) and to segregate the duplicated chromosomal DNA equally to two daughter cells [during mitosis (or M phase)] (Fig. 1). In addition to the molecular regulators that drive these processes, a monitoring circuitry ensures that S phase is completed before mitosis begins and *vice versa*. Early embryonic cell cycles exhibit rapidly alternating S and M phases without gap phases between them. This suggests that the gap phases seen in somatic cell cycles–G_1 separating the M and S phases, and G_2 separating the S and M phases–are not strictly essential for the correct operation of the cell cycle engine. It is intriguing to reflect on this point, because many of the G_1 phase regulators that prove so important in accelerating or braking the cell cycle engine of mammalian cells are encoded by nonessential genes, whose elimination from the germ line needs not lead to deleterious effects on organismal development.

G_1 phase is the interval in which cells respond to extracellular cues that ultimately determine whether cells will make the decision to replicate DNA and divide or, alternatively, to exit the cell cycle into a quiescent state (G_0). Once cells make the decision to begin DNA replication, they are irreversibly committed to complete the cycle, and the time late in G_1 phase at which this decision is made was designated the "restriction point" by Arthur Pardee (reviewed in Ref. 2; Fig. 1). When cells are stimulated by growth factors to enter the cycle from G_0, they generally require continuous mitogenic stimulation to be driven to the restriction point, after which mitogens can be withdrawn and cells will enter S phase and complete the cycle in their absence. Conversely, antiproliferative compounds, such as cytokines like transforming growth factor-β or drugs such as rapamycin, can only arrest the proliferation of cells that are progressing through G_1 phase but have not yet reached the restriction point. In mammalian cells, progression through the restriction point involves a series of events that lead to, but are distinct from, the firing of replication origins and that temporally precede the G_1-S transition by several hours (Fig. 1).

G1 Cyclins and cdks

In general, cell cycle transitions are controlled by cdks.[2] These holoenzymes contain both regulatory (cyclin) and catalytic (cdk) subunits but likely exist as higher order complexes that include additional proteins (see below). Restriction point control is mediated by two families of enzymes, the cyclin D- and E-dependent kinases. The D-type cyclins (D1, D2, and D3; Refs. 3–5) interact combinatorially with two distinct catalytic partners (cdk4 and cdk6; Refs. 6 and 7) to yield at least six possible holoenzymes that are expressed in tissue-specific patterns. Whereas cdk4 and cdk6 are relatively long-lived proteins, the D-type cyclins are unstable, and their induction, synthesis, and assembly with their catalytic partners all depend upon persistent mitogenic signaling. In this sense, the D-type cyclins act as growth factor sensors, forming active kinases in response to extracellular cues (reviewed in Ref. 8).

The mitogen-dependent accumulation of the cyclin D-dependent kinases triggers the phosphorylation of Rb, thereby helping to cancel its growth-repressive functions (6, 9, 10). Rb represses the transcription of genes whose products are required for DNA synthesis. It does so by binding transcription factors such as the E2Fs (reviewed in Ref. 11) and recruiting repressors such as histone deacetylases (12–14) and chromosomal remodeling SWI/SNF complexes (15) to E2F-responsive promoters on DNA. However, Rb phosphorylation by the G_1

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[2] The abbreviations used are: cdk, cyclin-dependent kinase; Rb, retinoblastoma protein; ARF, alternative reading frame; MEF, mouse embryo fibroblast.

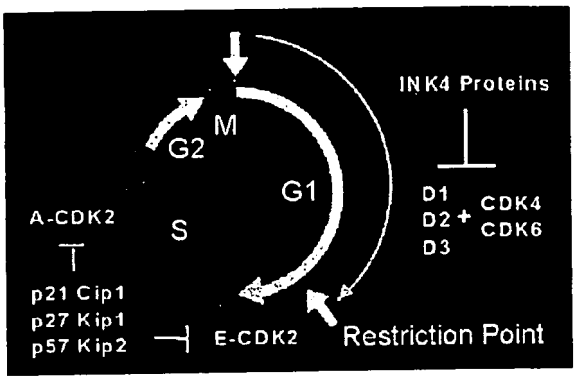

Fig. 1. G1 cdks and cdk inhibitors. Somatic cell cycles consist of alternating DNA synthetic (*S*) and mitotic (*M*) phases, separated by gap phases (*G1* and *G2*) as indicated. Mammalian cells respond to extracellular mitogens and antiproliferative cytokines from the time that they exit mitosis (*vertical arrow* at *top*) until they reach the restriction point, after which they can complete the cell division cycle in the absence of extracellular growth factors. Cyclin D-dependent kinases accumulate in response to mitogenic signals and initiate the phosphorylation of Rb, a process that is completed by cyclin E-cdk2. Once cells enter S phase, cyclin E is degraded and cyclin A enters into complexes with cdk2. INK4 proteins oppose the activities of the various cyclin D-dependent kinases, whereas Cip/Kip proteins specifically inhibit cyclin E-cdk2 and cyclin A-cdk2.

cdks disrupts these interactions (15, 16), enabling untethered E2Fs to function as transcriptional activators (Fig. 2). Apart from a battery of genes that regulate DNA metabolism, E2Fs induce the cyclin E and A genes. Cyclin E enters into a complex with its catalytic partner cdk2 (17–20) and collaborates with the cyclin D-dependent kinases to complete Rb phosphorylation (Fig. 2; Refs. 21–26). This shift in Rb phosphorylation from mitogen-dependent cyclin D-cdk4/6 complexes to mitogen-independent cyclin E-cdk2 accounts in part for the loss of dependency on extracellular growth factors at the restriction point. Cyclin E-cdk2 also phosphorylates substrates other than Rb, and its activity is somehow linked to replication origin firing (27, 28). The activity of the cyclin E-cdk2 complex peaks at the G_1-S transition, after which cyclin E is degraded and replaced by cyclin A (Fig. 1).

Cdk Inhibitors: The Cip/Kip Family

The actions of cdk2 are opposed by the *Cip/Kip* family of polypeptide inhibitors that includes $p21^{Cip1}$ (29–31), $p27^{Kip1}$ (32–34), and $p57^{Kip2}$ (Refs. 35 and 36; reviewed in Ref. 37; Fig. 1). In quiescent cells, the levels of $p27^{Kip1}$ are generally high. However, as cells enter cycle and accumulate cyclin D-dependent kinases, the Cip/Kip proteins are sequestered in complexes with cyclin D-dependent cdks (Fig. 2). Although it was initially assumed that the Cip/Kip proteins would inhibit both cdk4/6 and cdk2, we now recognize that the Cip/Kip-bound cyclin D-dependent enzymes remain catalytically active (38–41). Even more surprisingly, it turns out that Cip/Kip proteins are required for the assembly of the active cyclin D-dependent holoenzymes (Refs. 40 and 41; Fig. 2). In cycling cells, virtually all of the $p27^{Kip1}$ molecules remain associated with cyclin D-cdk complexes. However, mouse embryo fibroblasts taken from animals lacking both the *Kip1* and *Cip1* genes–$p57^{Kip2}$ is not synthesized in these cells–express no detectable cyclin D-dependent kinase activity and still have relatively unperturbed cell cycle transit times (41). In this setting, the levels of cyclin E-cdk2 activity are greatly increased and are apparently sufficient to phosphorylate Rb. Together, these data point to a second noncatalytic role of the cyclin D-dependent kinases, *i.e.*, the mitogen-dependent accumulation of cyclin D-dependent kinases sequesters Cip/Kip proteins, thereby facilitating cyclin E-cdk2 activation (reviewed in Ref. 42). This complements the Rb-E2F transcriptional program (see above) and helps make the appearance of cyclin E-cdk2 activity contingent upon accumulation of cyclin D-cdk4/6-Cip/Kip complexes (Fig. 2).

Once cyclin E-cdk2 is activated, it phosphorylates $p27^{Kip1}$. This converts $p27^{Kip1}$ to a form that is recognized by ubiquitin ligases and is targeted for destruction in proteasomes (43–47). Therefore, cyclin E-cdk2 antagonizes the action of its own inhibitor (Fig. 2). It follows that once cyclin E-cdk2 is activated, unbound $p27^{Kip1}$ is rapidly degraded, contributing to the irreversibility of passage through the restriction point. If cells are persistently stimulated with mitogens, cyclin D-dependent kinase activity remains high in subsequent cycles, $p27^{Kip1}$ levels stay low, and virtually all of the $p27^{Kip1}$ can be found in complexes with the cyclin D-cdks. However, when mitogens are withdrawn, cyclin D is rapidly degraded, and previously sequestered Cip/Kip proteins are mobilized to inhibit cyclin E-cdk2, thereby arresting progression usually within a single cycle.

The *Kip1* gene was not initially thought to be a tumor suppressor, because both copies of the gene were not found to be deleted or silenced in tumor cells. Yet, there is now compelling evidence that *Kip1* is haplo-insufficient for tumor suppression, with loss of only one copy of the gene being sufficient to contribute to cancer (48). In retrospect, we might rationalize this finding through the realization that Cip/Kip proteins are essential for the formation of cyclin D-dependent holoenzymes, although, at least experimentally, $p21^{Cip1}$ can functionally replace $p27^{Kip1}$ in this regard. Still, low levels of $p27^{Kip1}$ (which can be associated with monoallelic *Kip1* deletions in tumor cells) combined with high levels of cyclin E are generally indicative of reduced long-term survival in various forms of cancer. This has been well documented in breast cancer, where the levels of $p27^{Kip1}$ and cyclin E in primary tumors have greater prognostic power than other markers (49, 50). It is particularly important in women without apparent lymph node involvement, in whom the choice of therapy critically depends on strongly predictive markers of this type.

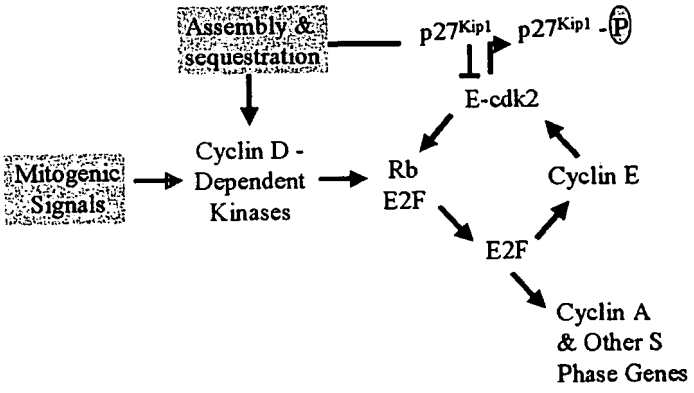

Fig. 2. Restriction point control and the G_1-S transition. As cells enter the division cycle from quiescence, the assembly of cyclin D-dependent kinases in response to mitogenic signals requires Cip/Kip proteins, which are incorporated into catalytically active holoenzyme complexes. The cyclin D-dependent kinases initiate Rb phosphorylation, releasing E2F from negative constraints and facilitating activation of a series of E2F-responsive genes, the products of which are necessary for S-phase entry. Activation of cyclin E by E2F enables formation of the cyclin E-cdk2 complex. This is accelerated by the continued sequestration of Cip/Kip proteins into complexes with assembling cyclin D-cdk complexes. Cyclin E-cdk2 completes the phosphorylation of Rb, further enabling activation of E2F-responsive genes, including cyclin A. Cyclin E-cdk2 also phosphorylates $p27^{Kip1}$, targeting it for ubiquitination and proteasomal degradation. The initiation of the self-reinforcing E2F transcriptional program together with degradation of $p27^{Kip1}$ alleviates mitogen dependency at the restriction point and correlates with the commitment of cells to enter S phase. In subsequent cycles, cyclin D-dependent kinases remain active as long as mitogens are present, and levels of $p27^{Kip1}$ remain low. All $p27^{Kip1}$ in cycling cells is complexed with cyclin D-cdk complexes. Mitogen withdrawal results in cyclin D degradation, liberating $p27^{Kip1}$ from this latent pool. The resulting inhibition of cyclin D- and E-dependent kinases leads to cell cycle arrest, usually within a single cycle.

Cdk Inhibitors: The INK4 Family

Another class of cdk inhibitors, the so-called INK4 proteins (named for their ability to inhibit cdk4), specifically target the cyclin D-dependent kinases (reviewed in Refs. 37 and 51; Fig. 1). INK4 proteins sequester cdk4/6 into binary cdk-INK4 complexes, liberating bound Cip/Kip proteins, and thereby indirectly inhibiting cyclin E-cdk2 to ensure cell cycle arrest (reviewed in Ref. 42). The ability of INK4 proteins to arrest the cell cycle in G_1 phase depends upon the presence of a functional Rb protein, implying that by inhibiting cyclin D-dependent kinases, Rb remains hypophosphorylated and able to repress transcription of S-phase genes (52–54). Note that disruption of cyclin D-cdk complexes and release of bound Cip/Kip proteins is insufficient to inhibit cyclin E-cdk2 in Rb-negative cells. This is likely attributable to the fact that cyclin E-cdk2 activity is normally under Rb-E2F control (Fig. 2), so that cells lacking Rb exhibit greatly elevated cyclin E-cdk2 kinase activity. This enables a conceptually simplified view of the "Rb pathway": INK4 proteins — cyclin D-dependent kinases — Rb — E2Fs → S phase entry.

To date, four INK4 proteins have been identified. These include the founding member $p16^{INK4a}$ (55) and three other closely related genes designated $p15^{INK4b}$ (56), $p18^{INK4c}$ (52, 57), and $p19^{INK4d}$ (57, 58). In humans, *INK4a* and *INK4b* are closely linked on the short arm of chromosome 9 (59), whereas *INK4c* maps to chromosome 1 and *INK4d* maps to chromosome 19. In mice, the *INK4c* and *INK4d* genes are expressed in stereotypic patterns in different tissues during development *in utero*, whereas *INK4a* and *INK4b* expression has not been detected prenatally (60). Gene disruption experiments in mice have revealed no overt effects of *INK4b* or *INK4d* loss (61). In contrast, mice lacking *INK4c* are similar to those lacking *Kip1* in the sense that they have organomegaly and pituitary tumors (62). "Pure" *INK4a*-null mice have not yet been produced (see below). However, one report has provided evidence that inbred BALB/c mice contain defective *INK4a* alleles that encode $p16^{INK4a}$ proteins incapable of inhibiting cyclin D-dependent kinases (63). At face value, the collective data argue that disabling single INK4 family members does not particularly increase the rate of spontaneous tumor development in mice.

Nonetheless, there is compelling evidence that *INK4a* loss-of-function occurs frequently in human cancers (reviewed in Ref. 51). In some familial melanomas, for example, one defective copy of *INK4a* is inherited, whereas the second is lost in tumor cells, the reduction to homozygosity being a classic feature of a tumor suppressor gene (59). In many forms of sporadic cancer, *INK4a* function is also lost (51). For example, virtually all pancreatic carcinomas exhibit *INK4a* defects. As might be expected, the loss of *INK4a* represents only one of several ways in which the Rb pathway can be disabled. In glioblastomas, CDK4 is frequently amplified, and *INK4a* function is lost in other cases. In small cell lung cancer, ~85% of tumors sustain Rb loss, whereas the remaining tumors exhibit *INK4a* loss-of function (10%) or cyclin D amplification (5%; reviewed in Refs. 51 and 64). A remaining puzzle is why other members of the INK4 gene family are not similarly targeted in human tumors. It therefore seems that *INK4a* plays a special role in tumor surveillance in humans. Whatever the reason for the preferential involvement of $p16^{INK4a}$, the available data have led to the reasonable speculation that disruption of the Rb pathway is part of the life history of many, if not all, human tumor cells (reviewed in Ref. 64).

The ARF Tumor Suppressor

Surprisingly, the *INK4a* gene encodes a second potent tumor suppressor (65, 66). The sequences encoding $p16^{INK4a}$ are embodied in three exons (designated 1α, 2, and 3), which specify an mRNA transcript of ~1 kb. In the human and mouse genomes, an alternative first exon (designated 1β) lies 15–20 kb upstream of the $p16^{INK4a}$ coding sequences, and its RNA is spliced to the exon 2 and exon 3 RNA segments to yield a second ~1 kb "β mRNA" whose 5′ end differs from the α transcript (65, 67–69). Alternative promoters located 5′ of exons 1α and 1β govern the independent production of the two mRNAs. The unusual feature is that the initiation codons within exons 1α and 1β are in different reading frames and, when spliced to the same sequences in exon 2, encode two distinct proteins that bear no relationship to one another (65). In the mouse, the ARF protein is represented by 64 amino acids encoded by exon 1β and 105 amino acids specified by exon 2. Mouse $p19^{ARF}$ is a highly basic protein that, when overexpressed, can cause cell cycle arrest in both the G_1 and G_2 phases of the cell cycle (65). Its human counterpart ($p14^{ARF}$) contains fewer exon 2-coded amino acids and is of lower molecular mass, but it has the same ability to induce cell cycle arrest.

Mice containing disrupted *INK4a/ARF* exon 2 sequences (70) or lacking only the *ARF* exon 1β sequences (66) are highly tumor prone and die of cancers within 15 months of age. The most predominant tumors are sarcomas, followed by lymphomas, carcinomas, and tumors of the central nervous system (71). *ARF+/−* heterozygotes develop tumors after a considerably longer latency, and the tumor cells lose the wild-type *ARF* allele, as is characteristic of a classical tumor suppressor gene. When MEFs of *INK4a/ARF* or *ARF*-null animals are explanted into culture and passaged on a defined 3T3 protocol, the cells do not senesce but rather continue to proliferate as established cell lines (66, 70). Normally, primary MEFs are generally resistant to transformation by oncogenic *Ras* and require the introduction of a so-called immortalizing oncogene, such as adenovirus *E1A* or *Myc*, to undergo transformation (72, 73). However, like established rodent fibroblast lines, *ARF*-null cells can be transformed by oncogenic *Ras* alone (66, 70). In these respects, *ARF*-null MEFs are similar to *p53*-deficient mouse fibroblasts, which are also immortal and can be transformed by Ras without a requirement for Myc or E1A. Moreover, spontaneously immortalized cells derived from a 3T3 protocol contain either mutations in the *p53* gene (80%) or exhibit bi-allelic *ARF* loss (the remaining 20%; Refs. 66 and 74). Together, these data suggested that ARF and p53 functioned in the same biochemical pathway.

p53 is a homotetrameric transcription factor that induces either cell cycle arrest or apoptosis, depending on the biological setting (reviewed in Refs. 75 and 76). Introduction of *ARF* into cells results in p53-dependent cell cycle arrest, indicating that ARF acts "upstream" of p53 (66). Cells lacking p53 alone are refractory to ARF-induced arrest, and in this setting, ARF protein expression is greatly increased. This suggests that p53 suppresses *ARF* expression through negative feedback, and consistent with this interpretation, reintroduction of p53 into these cells returns ARF protein expression to lower levels (Refs. 77 and 78; Fig. 3). ARF stabilizes p53 by antagonizing the p53-negative regulator Mdm2 (77–80). Mdm2 binds to the transactivation domain of the p53 tetramer to inhibit p53-dependent gene expression (81, 82), and it also manifests a ubiquitin ligase activity that appears to target p53 for proteasomal degradation (83). Intriguingly, Mdm2 is itself a p53-responsive gene that normally acts in feedback control to terminate the p53 response (Refs. 84 and 85; Fig. 3). ARF can interfere with all of the known functions of Mdm2, including its ability to: (*a*) block p53 transcription (77–79); (*b*) to ubiquinate p53 (86); and (*c*) to enforce p53 transport into the cytoplasm (87–89), where it is degraded in proteasomes (90–92).

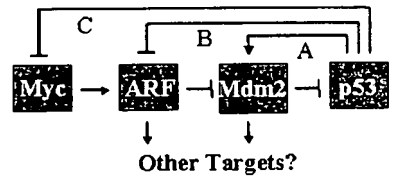

Fig. 3. The ARF-Mdm2-p53 pathway. In response to proteins such as Myc and E2F1 (not shown), ARF protein accumulates and antagonizes the activity of Mdm2. The resulting accumulation of p53 leads to cell cycle arrest or apoptosis, depending on the biological setting. Mdm2 is a p53-responsive gene (pathway *A*) whose p53-dependent accumulation helps to terminate the p53 response. In addition, p53 negatively regulates both ARF (pathway *B*) and Myc (pathway *C*) through as yet unknown mechanisms. ARF likely interacts with targets other than Mdm2, whereas Mdm2 may also functionally interact with proteins other than p53. [This figure is adapted from Eischen *et al.* (100) and is reprinted with permission from *Genes & Development.*]

ARF Connects Rb and p53

ARF expression is activated by abnormal mitogenic signals induced by overexpression of oncoproteins such as Myc (93), E1A (94), E2F1 (95), Ras (96), and v-Abl (97). In this manner, ARF serves to connect the Rb pathway with Mdm2 and p53 (Fig. 4). ARF acts as a fuse to "gate" inappropriate mitogenic signals flowing through the cyclin D-cdk –Rb –E2F circuit, inducing p53 under conditions in which abnormal proliferative signals are generated. This mode of cell-autonomous tumor surveillance diverts cells that have received an oncogenic insult to undergo p53-dependent growth arrest or apoptosis, thereby preventing incipient cancer cells from emerging as overt tumors (reviewed in Ref. 98).

But, if genes like Myc and E1A can induce *ARF* and p53 to trigger growth arrest or cell death, how can these same genes immortalize normal cells and collaborate with oncogenic Ras to transform them? A reasonable hypothesis is that Myc and E1A overexpression, by inducing both cell proliferation and compensating p53-dependent apoptosis, selects for resistant cells that have sustained mutations in the ARF-Mdm2-p53 pathway and that can now be transformed by oncogenic *Ras* alone. To test this idea, primary MEFs were infected with a high titer *Myc* retrovirus, and Myc-induced apoptosis was enforced by depriving the infected cells of serum-containing survival factors. Rare surviving cells were then recloned, expanded as colonies, and genotyped for *p53* mutations and/or *ARF* loss. Strikingly, all such colonies lost the function of p53 or ARF but not both (93). In short, ARF normally acts to protect cells from Myc overexpression by facilitating Myc-induced, p53-dependent apoptosis. Cells corrupted in the ARF-Mdm2-p53 pathway are resistant to Myc-induced killing, enabling Myc to act as a pure growth promoter in this setting.

Studies using animal models support the view that ARF protects cells against Myc-induced tumorigenesis. Mice bearing an Eμ-*Myc* transgene, in which Myc expression is driven by an immunoglobulin heavy chain enhancer, develop Burkitt-type B cell lymphomas with a mean latency of ~30 weeks, and all die of the disease by 1 year of age (99). In the early stages before overt tumors arise, the B-cell compartment of these animals exhibits hyperproliferation, which is balanced by increased apoptosis. When tumors arise, however, the apoptotic index is greatly diminished (100, 101). About 75% of these lymphomas had lesions (*p53* or *ARF* loss, or Mdm2 overexpression) that disabled the ARF-Mdm2-p53 pathway. In addition, when Eμ-*Myc* mice were crossed onto an *ARF*+/− background, tumor progression was greatly accelerated (mean latency, 12 weeks) and 80% of the resulting tumors had lost the wild-type *ARF* allele. Even more strikingly, on an *ARF*-null (100) or *INK4a-ARF*-null (101, 102) background, Eμ-*Myc* transgenic animals all died of highly aggressive lympholeukemias by only 5–6 weeks of age. By contrast, Rb loss of function did not significantly accelerate Eμ-*Myc*-induced disease (101). Most tumors that sustain *ARF* or *p53* mutations do not respond to therapies that can cure mice in earlier stages of lymphoma development (101). Therefore loss of the ability of ARF to modulate the p53 response connotes a poor prognosis, even in those tumors retain wild-type *p53*.

These data imply that by dynamically resetting the effective Mdm2 threshold, ARF reduces the ability of p53 to function in tumor suppression. Consistent with this view, loss of *ARF* makes cells relatively resistant to apoptosis induced by ionizing radiation or cyclophosphamide (94) and can sensitize cells to polyploidy induced by microtubule inhibitors (103). *ARF* loss, like *p53* mutation, can also rescue cells lacking the *Atm* gene from undergoing premature senescence in culture (104), indicating that *ARF* loss modulates the Atm-dependent DNA damage checkpoint. Thus, although DNA damage signals do not appear to activate *ARF per se* (Fig. 4), ARF loss modulates p53 function in such a way as to diminish its accumulation in response to genotoxic stress.

Although the most parsimonious interpretation is that ARF functions in a linear pathway by harnessing the ability of Mdm2 to neutralize p53, there are several reasons to believe that the ARF-Mdm2-p53 pathway has alternative branch points. One line of argument concerns the feedback loops, in which p53 can both induce Mdm2 and repress ARF levels (Fig. 3). The biochemical basis for these connections remains unclear. Moreover, in some *Myc*-induced lymphomas, perturbations were observed that affected expression of more than one gene in the pathway (100). For example, a significant

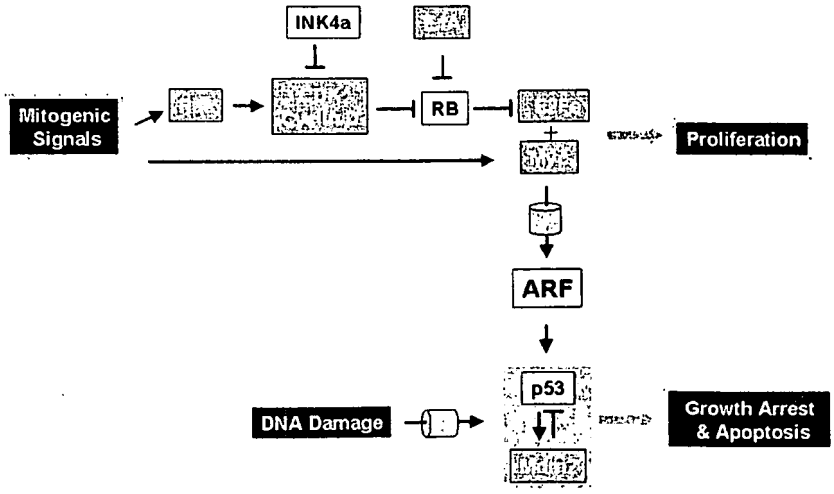

Fig. 4. ARF tumor surveillance. When induced by inappropriate mitogenic signals, ARF antagonizes Mdm2 to activate p53. Hence, hyperproliferative signals are countered by ARF-dependent p53 induction, which diverts incipient cancer cells to undergo growth arrest and/or apoptosis. Loss of the ARF checkpoint (indicated by the *vertical barrel*) subverts this form of cell-autonomous tumor surveillance and allows proteins such as Ras, Myc, E1A, and E2F to function as "pure" proliferation enhancers. DNA damage signals engage various ARF-independent signaling pathways (shown collectively by the *horizontal barrel*) that stabilize p53, most commonly by inducing posttranslational modifications in p53 and/or Mdm2 that prevent their interaction. Although ARF is not directly activated by ionizing radiation or various genotoxic drugs, it is still a potent modifier of the DNA damage response. ARF induction sensitizes cells to DNA damage signals; conversely, ARF loss increases the Mdm2 response and severely dampens the p53 response. All proteins enclosed by *shaded boxes* are potential oncogenes, whereas those illustrated by *unfilled boxes* are tumor suppressors. [This figure is adapted from Sherr (98) and is reprinted with permission from *Genes & Development.*]

fraction of lymphomas exhibited both *ARF* loss and Mdm2 overexpression, implying that both genes can contribute independently to tumor formation. One possibility is that Mdm2 encodes different truncated isoforms, whose as yet undetermined functions may differ from the full-length molecule. At least in principle, ARF might act on targets other than Mdm2, and Mdm2 in turn might regulate proteins other than p53 (Fig. 3). Indeed, there are precedents for the latter, based on reported interactions of Mdm2 with other p53 family members (105), Rb (106, 107), p300 (108), and even E2F1 (109). Much more work is required to critically evaluate these possibilities. Still, it seems evident that disruption of the ARF-Mdm2-p53 pathway occurs frequently in cancers. In humans, *p53* is itself mutated in >50% of cancers, whereas *ARF* loss and Mdm2 overexpression occur in a high fraction of the remaining cases. Hence, disruption of ARF, Mdm2, and p53, like mutations in the $p16^{INK4a}$-cyclin D/cdk4-Rb pathway, again seem to be part of the life history of cancer cells, irrespective of patient age or tumor type.

ARF: In Search of Biochemical Function

ARF is a highly basic protein that localizes to the nucleolus (65, 79, 89). When induced or overexpressed, ARF binds to Mdm2 and imports it into the nucleolus, thereby allowing *p53* to accumulate in the nucleoplasm (89, 110, 111). Recently, ARF was found to bind to a central region of Mdm2 to a segment distinct from Mdm2's nuclear import and export signals, its NH_2-terminal *p53* binding domain, and the COOH-terminal RING domain, the integrity of which is required for E3 ubiquitin ligase activity (111). Both the human and mouse ARF proteins contact Mdm2 through two independent binding sites that are separated by spacer elements of different lengths in the two proteins. In mouse $p19^{ARF}$, the two Mdm2 binding sites cluster in the ARF NH_2 terminus within amino acids 1–37 (111). Segments containing amino acids 1–14 and 26–37 are responsible for cooperative binding and induce an allosteric change in Mdm2 that facilitates its nucleolar import. Interestingly, a cryptic localization signal within the COOH-terminal Mdm2 RING domain contributes to the nuclear import of the ARF-Mdm2 complex. Mutations within this region prevent Mdm2 nucleolar import and instead result in ARF sequestration by Mdm2 in the nucleoplasm (110, 111). The fact that Mdm2 RING domain mutants can oppose the activity of ARF implies that the ARF-Mdm2 interaction is bidirectional, with each protein having a potential to cancel activities of the other.

Although the spacing between the Mdm2 binding domains in the human $p14^{ARF}$ protein is greater than that in mouse $p19^{ARF}$ (88), human or mouse ARF mutants that either do not interact properly with Mdm2 or colocalize Mdm2 to the nucleolus are impaired in arresting cell proliferation (89, 110, 111). To date, these functional data suggest that the ability of ARF to sequester Mdm2 correlates with *p53*-dependent cell cycle arrest. However, it is formally possible that ARF might also antagonize Mdm2 in the nucleoplasm (88). These findings raise interesting questions about the *in vivo* activities of ARF. Is the primary role of ARF to sequester Mdm2 from p53 (89, 110, 111), to interfere with Mdm2-catalyzed ubiquitination (83), to prevent Mdm2 from enforcing p53 nuclear export (87–89, 110, 111), or all of the above?

Conclusions and Future Prospects

In summary, studies over the last decade have indicated that most human cancer cells sustain mutations that affect the functions of Rb and *p53*, either by disabling these genes directly or by targeting genes that act epistatically to prevent their proper function. The *INK4a/ARF* locus surprisingly encodes two products that affect both Rb and *p53*, and the rationale for nature's design of these overlapping tumor suppressors continues to pose a puzzle. An implication may be that the activities of *INK4a* and *ARF* are somehow coregulated through their proximity in the genome, although much of the data collected thus far argue against this interpretation. There is clearly more to learn here.

If, in fact, it is true that disabling the Rb and p53 pathways is a hallmark of cancer, then the most efficacious treatment would be to restore their functions. The inability to specifically target genes to tumor cells and to properly regulate their expression makes "gene therapy" impractical. Novel therapeutics will likely need to target ancillary pathways. Can we take advantage of weaknesses in tumors lacking Rb and/or p53 to selectively kill them? One rationale is based on the concept of "synthetic lethality" in yeast, in which disruption of one gene–in this case, Rb and/or p53–might sensitize cells to disruption of another pathway while sparing cells that retain either one of the two functions. Cyclin A-cdk2 activity is required to terminate E2F function in S phase, and blocking this function triggers apoptosis (112, 113). One idea, then, is that cells that lack Rb and *p53* checkpoints might prove more sensitive than normal cells to cdk inhibitors (114), which are now being widely developed. The recent realization that cells lacking a p53-inducible nuclear subunit of ribonucleotide reductase may rely on a cytoplasmic form of this enzyme to resist drug-induced genotoxic damage (115) may provide another opportunity for targeted therapy. Others have speculated that if specific inhibitors of the cytoplasmic form of ribonucleotide reductase could be developed, these might selectively sensitize cells with mutant p53 to DNA-damaging chemotherapeutic agents (116). Yet another approach would be to activate the apoptotic machinery downstream of the sensory signals that normally lead to *p53*-dependent activation, *e.g.*, by activating death-inducing receptors that couple to caspases (117). An article of faith is that a better understanding of cancer cells will lead to new drug targets and novel therapeutic approaches–that good science will lead to good medicine. Here, again, I quote from Thomas:

"It is much more difficult to be convincing about ignorance concerning disease mechanisms than it is to make claims for full comprehension, especially when the comprehension leads, logically or not, to some sort of action. When it comes to serious illness, the public tends, understandably, to be more skeptical about the skeptics, more willing to believe the true believers. It is medicine's oldest dilemma, not to be settled by candor . . . What it needs is a lot of time and patience, waiting for the science to come in, as it has in the past, with the solid facts" (118).

We should be optimistic that the learning curve is accelerating.

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